# COMPARATIVE EVALUATION OF PROLIFERATION & DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS AND RADIOPACITY USING PRF, PRF+ 50WT% NANOHYDROXYAPATITE, PRF+ 50WT%DENTIN CHIPS

## - AN IN VITRO STUDY

*A Dissertation submitted*
*in partial fulfillment of the requirements*
*for the degree of*

**MASTER OF DENTAL SURGERY**
**BRANCH – IV**
**CONSERVATIVE DENTISTRY AND ENDODONTICS**


**THE TAMILNADU DR. MGR MEDICAL UNIVERSITY**
**CHENNAI – 600 032**
**2015 – 2018**

# DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled**"COMPARATIVE EVALUATION OF PROLIFERATION & DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS AND RADIOPACITY USING PRF, PRF+ 50WT% NANOHYDROXYAPATITE, PRF+ 50WT%DENTIN CHIPS - AN IN VITRO STUDY"**is a bonafide and genuine research work carried out by me under the guidance of **Dr.M.KAVITHA M.D.S., Professor& HOD,** Department Of Conservative Dentistry and Endodontics, Tamil Nadu Government Dental College and Hospital, Chennai-600003.

**DR. GIRIJA. K**

## CERTIFICATE BY GUIDE

This is to certify that **Dr. GIRIJA. K,**Post Graduate student (2015-2018) in the Department of Conservative Dentistry and Endodontics, TamilNadu Government Dental College and Hospital, Chennai- 600003 has done this dissertation titled **"COMPARATIVE EVALUATION OF PROLIFERATION & DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS AND RADIOPACITY USING PRF, PRF+ 50WT% NANOHYDROXYAPATITE, PRF+ 50WT%DENTIN CHIPS - AN IN VITRO STUDY"**under my direct guidance and supervision in partial fulfillment of the regulations laid down by the Tamil Nadu Dr.M.G.R Medical University Chennai -600032, for M.D.S., Conservative Dentistry and Endodontics (Branch IV) Degree Examination .

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**Professor & HOD**

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**Department of Conservative Dentistry and Endodontics.**

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**ENDORSEMENT BY HEAD OF THE DEPARTMENT /**

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Above all I pray and thank **THE ALMIGHTY GOD** for His continuous grace, giving me the strength and courage to complete this monumental task.

# DECLARATION

| | |
|---|---|
| TITLE OF DISSERTATION | **"COMPARATIVE EVALUATION OF PROLIFERATION & DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS AND RADIOPACITY USING PRF, PRF+ 50WT%NANOHYDROXYAPATITE, PRF+ 50WT%DENTIN CHIPS - AN IN VITRO STUDY "** |
| PLACE OF THE STUDY | Tamil Nadu Government Dental College & Hospital, Chennai- 3. |
| DURATION OF THE COURSE | 3 YEARS |
| NAME OF THE GUIDE | DR. M. KAVITHA |
| HEAD OF THE DEPARTMENT | DR. M. KAVITHA |

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This agreement herein after the "Agreement" is entered into on this day Jan 2018 between the Tamil Nadu Government Dental College and Hospital represented by its **Principal** having address at Tamil Nadu Government Dental College and Hospital, Chennai - 600 003, (hereafter referred to as, 'the college')

And

**Mrs. Dr. M. Kavitha** aged 47 years working as **Professor & HOD** in Department of Conservative Dentistry & Endodontics at the college, having residence address at 69/4,Mettu street, Ayanavaram, Chennai-23 (herein after referred to as the Principal Investigator')

And

**Miss. Dr.Girija. K** aged 29 years currently studying as **Post Graduate student** in Department of Conservative Dentistry & Endodontics, Tamil Nadu Government Dental College and Hospital, Chennai -3 (herein after referred to as the PG student and coinvestigator').

Whereas the PG student as part of her curriculum undertakes to research on **"COMPARATIVE EVALUATION OF PROLIFERATION & DIFFERENTIATION OF HUMAN DENTAL PULP STEM CELLS AND RADIOPACITY USING PRF, PRF+ 50WT% NANOHYDROXYAPATITE, PRF+ 50WT%DENTIN CHIPS - AN IN VITRO STUDY "**for which purpose the Principal Investigator shall act as principal investigator and the college shall provide the requisite infrastructure based on availability and also provide facility to the PG student as to the extent possible as a Co-investigator.

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In witness where of the parties herein above mentioned have on this day, month and year herein above mentioned set their hands to this agreement in the presence of the following two witnesses.

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**Witnesses** **Student Guide**

1.

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TELEPHONE : 044-253403343
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Ref No: R. C. NO: 0420/DE/2016 date : 31-10-2016
Sub: IEC review of the research proposals,

Title of the work: **Comparative evaluation of proliferation and differentiation of human dental pulp stem cells and radiopacity using PRF, PRF+50 wt% nano hydroxyapatite, PRF+50wt% dentin chips- An invitro study**

Investigator: Dr. Girija.K
II year MDS

Department : Department of Conservative dentistry and Endodontics
Tamil Nadu Govt. Dental College & Hospital , Chennai-3

Thank you for submitting your research proposal , which was considered at the Institutional Ethics Committee meeting held on the 30-09-2016, at TN Govt. Dental College. The documents related to the study referred above were discussed and the modifications done as suggested and reported to us through your letter On 25-11-2016 have been reviewed. The decision of the members of the committee , the secretary and the Chairperson IEC of TN Govt. Dental College is here under:

| | |
|---|---|
| Approved | Approved and advised to proceed with the study |
| Approved with suggestions | ---------- |
| Revision | ---------- |
| Rejected | ---------- |

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1. You should get detailed informed consent from the patients / participants and maintain confidentiality.
2. You should carry out the work without affecting regular work and without extra expenditure to the Institution or the Government.
3. You should inform the IEC, in case of any change of study procedure, site, and investigating guide.
4. You should not deviate from the area of work for which you have applied for ethical clearance.
5. You should inform the IEC immediately in case of any adverse events or serious adverse reactions. You should abide to the rules and regulations of the institution(s) .
6. You should complete the work within specific period and if any extension of time is required, you should apply for permission again to do the work.
7. You should submit the summary of the work to the ethical committee every 3 months and on completion of the work.
8. You should not claim any kind of funds from the institution for doing the work or on completion/ or for any kind of compensations.
9. The members of the IEC have the right to monitor the work without prior intimation.
10. Your work should be carried out under the direct supervision of the guide/ Professor.
11. The investigator and Guide should each declare that no plagiarism is involved, in this whole study and enclose the undertaking in dissertation/ thesis.

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**ABSTRACT**

**AIM** : The aim of this invitro study was to impart radiopacity to PRF using 2 bioactive radiopacifiers (Nanohydroxyapatite& dentin chips) and toassesss the cell proliferation, differentiation and mineralization of Human dental pulp stem cells induced by the modified PRF.

**MATERIALS AND METHODS**:PRFwasmodified with 50wt% of nanohydroxyapatite (nHA) and 50wt% of dentin chips (DC). The 5 samples of each group (Group1- Dentin disc, Group2- PRF, Group3 - PRF+ 50wt% nHA , Group4- PRF+ 50wt% DC) were digitally radiographed together with 8mm aluminium step wedge using DIGORA Software (for Windows 2.9.113.490). The aluminium equivalent of radiopacity obtained was compared with dentin disc (control). Further HDPSCs proliferation, differentiation and mineralization by the groups [(Group A- Control (DMSO), Group B- PRF, Group C- PRF+nHA, Group D- PRF+DC)]were assessed. MTT assay was used to assess cytotoxicity of the samples, ELISA was done to quantify the IL-6 & IL-8 cytokine expression & alizarin red assay was used to determine the mineralization induced. The odontoblasticdifferentiation of groups were evidenced by the protein expressions - DSPP, DMP1and STRO--1 was used for characterization of stem cells. These protein expressions (DSPP, DMP1, STRO-1) were quantified through western blot assay.

**RESULTS**: The mean aluminium equivalent radiopacity among each groups showed statistical significant results with $P < 0.05$.Group 3 (PRF+ 50wt% nHA) was able to achieve an aluminium equivalent radiopacity (1.51±0.089) better than Group 4 (0.97± 0.22 mmAl). The cell viability was more than 75% for all groups. IL-6 & IL-8 expression by Group D (PRF+50wt% DC) was higher compared to Group B (PRF) and Group C (PRF+ 50wt% DC). Group C (PRF+ 50wt% nHA) induced more minerlization nodules compared to other groups. The integrated density value for the DSPP and DMP-1 protein expression by Group C (PRF+ 50wt% nHA) and Group D(PRF+ 50wt% DC) was higher compared to Group B (PRF). STRO- 1 expression by Group C (PRF+ 50wt% nHA ) was highest among all the groups.

**CONCLUSION**: This study showed that the addition of bioactive radiopacifiersinto PRF were able to impart radiopacity and this combination has a synergistic effect on the stimulation of odontoblastic differentiation of HDPCs, hence inducing mineralization.

**KEY WORDS:** Nanohydroxyapatite, Dentin chips,Human Dental Pulp Stem Cells, Bioactive radiopacifiers, DIGORA Software, Aluminium equivalent radiopacityMTT assay, ELISA, Alizarin red assay, Western blot assay

## CONTENTS

## ABBREVIATIONS USED

| | |
|---|---|
| nHA | Nano-hydroxyapatite |
| DC | Dentin chips |
| PRF | Platelet Rich Fibrin |
| PRP | Platelet Rich Plasma |
| HDPSC | Human Dental Pulp Stem Cells |
| HA/TCP | Hydroxyapatite/Tricalcium phosphate |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| DMSO | Dimethyl Sulfoxide |
| BSA | Bovine Serum Albumin |
| SDS | Sodium dodecyl sulfate |
| NBT | Nitrobluetetrazolium |
| BCIP | 5 Bromo-4-chloro-3-indolyl phosphate |
| PBS | Phosphate Buffer Solution |
| DSPP | Dentin Sialophosphoprotein |

| | |
|---|---|
| DMP1 | Dentin Matrix Protein 1 |
| DMEM | Dulbecco's Modified Eagle Medium |
| FBS | Fetal Bovine Serum |
| STV | Saline Trypsin Versene |
| ARS | Alizarin Red S |
| $DiH_2o$ | De-ionized water |
| TMB | 3,3',5,5'-Tetramethylbenzidine |
| RDM | Radiographic density of material |

# LIST OF TABLES

# LIST OF GRAPHS

## INTRODUCTION

The modern exploration of regenerative dentistry has added impact into the field of molecular biology. The understanding of the molecular and cellular bases for regeneration and tissue repair lead to development of newer biologically based strategies for the successful clinical treatment. Currently the studies have been focussed on the use of an autogenous material that provides an osteoconductive scaffold along with growth factors to stimulate patient's own cells towards a regenerative response. In a quest to find a single biomaterial which promotes fast healing and maximum tissue regeneration, platelet concentrates from the peripheral blood was identified to provide predictable outcomes.

In an attempt to isolate those platelet concentrates using different techniques , geared to simplified procedure where a second generation Platelet concentrate, called Platelet rich fibrin (PRF) was developed , which was reported by Choukroun et al (2001)[20]. It consists of an autologous leukocyte-platelet-rich fibrin matrix composed of tetramolecular structure, with cytokines, growth factors, platelets and stem cells within it, which acts as a biodegradable scaffold and accelerates soft as well as hard tissue healing.

The second generation platelet concentrates were able to overcome disadvantages of first generation; platelet rich plasma (PRP). It outweighed in aspects of biological, physical, and mechanical properties. This Platelet rich fibrin which is formed through natural and progressive polymerization doesn't require anticoagulant bovine thrombin, thus reducing chances of cross infection[8]. Therefore, for successful

preparation of PRF, speedy blood collection and immediate centrifugation before the initiation of clotting cascade is absolutely essential[20].

It has made its noteworthinesss in various field of science especially in clinical applications of regenerative endodontics such as pulp capping, apexogenesis, revascularisation, and bone regeneration. Studies have shown that PRF can be used as a scaffolding material in an infected necrotic immature tooth for pulpal regeneration and tooth revitalization[5]. The potential theory behind the success of the use of PRF for regeneration of open apex could be attributed to a study conducted by Huang et al, who concluded that the PRF can induce proliferation of Human dental pulp cells and increases the protein expression of these dental pulp cells to differentiate into odontoblasts like cells.[34]

The combination of PRF membrane as a matrix and MTA in apexification procedures affirmed to be an effective alternative for creating artificial root-end barriers and to induce faster periapical healing in cases with large periapical lesions. Use of a membrane can prevent the extrusion of this material[8]. An in-vitro study showed that PRF is superior to PRP, considering the expression of alkaline phosphatase and induction of mineralization, caused markedly by release of TGF-β1and PDGF-AB[20].

But the clinical challenge associated with PRF is that it lacks radiopaque property, which will not be discernable on radiographs, thus making it difficult to identify the correct placement of PRF, especially during apexification procedure and in cases of regenerative procedures for an open apex teeth. To encounter this troubleshoot, we came up with a revitalizing concept of adding radiopacifier and to make it a traceable material.

Various commercially available radiopacifiers like Bismuth oxide, Iodoform, Zirconium oxide, Bismuth oxide were used in dental restorative material, root end filling material and root canal sealers to impart radiopacity. They are being heavy metals, has its limited availability and also may have cytotoxic effect to living tissue and resident stem cells. Thus any material added should be non-cytotoxic.

Studies have showed that PRF in conjunction with bone grafts promoted wound healing, Bone growth & maturation, Graft stabilization, Hemostasis and also improved the handling properties of graft materials. Nano –hydroxyapatite (nHA) which is an osteoconductive allograft material has been proven their role in proliferation and differentiation of mesenchymal cells into osteoblasts cells. This can be incorporated into a scaffold material, thus helps in forming a favourable hard tissue scaffold with the proper mechanical strength, hydrophilicity, and excellent osteoconductivity[23].

Dentin chips (DC), on the other hand, have been applied in a number of in vitro and in vivo studies as an apical filling material in root canal or graft material for filling osseous defect.[10,56,80] This material is not commercially available and for its preparation human teeth were autoclaved and crushed into chips. The presence of dentin promotes the formation of a calcified tissue similar to ossein and accelerates healing whilst inhibiting inflammatory reaction.[56]

Both Nano-hydroxyapatite (nHA) and Dentinchips (DC) are calcium phosphate based bioactive materials with osseoconductive property and are radiopaque in nature. These propitious qualities has made them as the material of choice for the present study to impart the radiopacity to PRF. The Organization for Standards, ISO

6876:2001 establishes 3 mm Al as the minimum radiopacity for root canal sealers.[81] According to the ANSI/ADA specification No. 571 , root canal sealers should be at least 2 mm Al more radiopaque than bone or dentin.[93] But there was a dilemma with the effect of these materials on the human dental pulp stem cells. Human Dental Pulp Stem Cells was chosen for the study because they are being of neural crest-derived cells which has an outstanding capacity of multifaceted differentiation , along with their non-tumorigenic phenotype, a high proliferation rate, a relative simplicity of extraction and culture, and their possibility of cryopreservation, which ultimately makes it possible to obtain patient-specific cell lines for use in an autologous cell therapy.[35]

However there are no in-vitro studies have been conducted to evaluate the radiopacity of PRF. This aspect has been taken into account where PRF was modified with bioactive radiopacifiers (Nano-hydroxyapatite & Dentin chips) and the aluminium equivalent of radiopacities were measured. Further the effect on Human Dental Pulp Stem Cell were assessed through cell viability, cell proliferation, odontoblastic differentiation and mineralization potential.

## AIM OF THE STUDY

The aim of the study is to compare the cell proliferation & differentiation of human dental pulp stem cells and radiopacity of PRF, PRF modified with 50wt% Nano-hydroxyapaptite, PRF modified with 50wt% dentin chips.

## OBJECTIVES

I. To evaluate the aluminium equivalent of radiopacity of PRF, PRF modified with 50 wt% Nano-hydroxyapatite (nHA) and PRF modified with 50 wt% Dentin chips (DC) by using DIGORA software.

II. To evaluate cell viability & cell proliferation of Human dental pulp stem cells and inflammation induced (IL-6 & IL-8 cytokine expression) by PRF, PRF modified with 50 wt% Nano-hydroxy apatite (nHA), PRF modified with 50wt% Dentin chips (DC) using MTT assay and ELISA respectively.

III. To evaluate mineralization potential of PRF, PRF modified with 50wt% Nano-hydroxyapatite (nHA), PRF modified with 50wt% Dentin chips (DC) on Human dental pulp stem cells by Alizarin red assay.

IV. To evaluate odontoblastic differentiation of Human dental pulp stem cells under the influence of PRF, PRF modified with 50wt% Nano-hydroxyapatite (nHA) , PRF modified with 50 wt% Dentin chips (DC) through the expression of DSSP and DMP-1 protein analysed using Western blot assay.

## REVIEW OF LITERATURE

## PLATELET RICH FIBRIN

**Ross R et al (1974)**[79] demonstrated that role of growth factors, derived from blood serum was responsible for cell proliferation. Difference was observed when dialyzed serum from clotted monkey blood ("blood serum") and dialyzed serum prepared from recalcified platelet-poor plasma ("plasma serum") used, where former one caused the proliferation of monkey arterial smooth muscle cells in culture. Addition of platelets and calcium to platelet-poor plasma increased the activity of plasma serum to the same level achieved with blood serum.

**Whitman et al (1997)**[99] described the preparation and use of platelet gel. It is derived from autologous blood collected in the immediate preoperative period, which contains a high concentration of platelets. It has been tried out successfully in the area of reconstructive oral and maxillofacial surgery, surgical repair of alveolar clefts and associated oral-antral/ oral-nasal fistulas, and adjunctive procedures related to the placement of osseointegrated implants and reported the advantages of PRP in regeneration as it enhances osteoprogenitor cells in the host bone and bone graft.

**Choukroun et al (2001)**[20] first used PRF specifically in oral and maxillofacial surgery, and is considered as second generation of platelet concentrate. PRF has able to overcome disadvantages of PRP, including easier preparation and not requiring anticoagulant bovine thrombin, which makes it strictly an autologous preparation.

**He L et al (2009)**[33] evaluated the effect of biologic characteristics of platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) on proliferation and differentiation of rat

osteoblasts. The exudates of PRP and PRF were collected at the time periods of 1, 7, 14, 21, and 28 days and were used to culture rat calvaria osteoblasts. The biologic characteristics of osteoblasts were analyzed in vitro for 14 days. PRP released the highest amounts of TGF-beta1 and PDGF-AB at the first day, followed by significantly decreased release at later time points. PRF released the highest amount of TGF-beta1 at day 14 and the highest amount of PDGF-AB at day 7. Exudates of PRP collected at day 1 and exudates of PRF collected at day 14 expressed maximum alkaline phosphatase (ALP) activity, though no significance was shown.

**Dohan Ehrenfest et al (2010)**[21] studied the cell composition and three-dimensional organization of PRF and evaluated the influence of different collection tubes (dry glass or glass-coated plastic tubes) and compression procedures (forcible or soft) on the final PRF-membrane architecture. Approximately 97% of the platelets and >50% of the leukocytes were concentrated in the PRF clot and showed a specific three-dimensional distribution, depending on the centrifugation forces. The fibrin network was very mature and dense. Moreover, there was no significant difference in the PRF architecture between groups using the different collection tubes and compression techniques.

**Huang et al (2010)**[34] conducted an investigation into the biological effects of PRF on human dental pulp cells. PRF was found to increase dental pulp cell proliferation as well as osteoprotegerin (OPG) expression in a time-dependent manner. Alkaline phosphatase (ALP) activity was also significantly up-regulated by PRF. These findings might serve as a basis for preclinical studies that address the role of PRF in reparative dentin formation.

**Keswani D, Pandey RK (2015)**[46] used freshly prepared PRF which was condensed into the canal of immature maxillary central incisor of the patient, until the cementoenamel junction, over which white MTA and permanent restoration has been done. At 12- and 15-month follow-up, the tooth responded positively to cold and an electric pulp test, and similarly to adjacent lateral incisor and canine teeth. Radiographic examination demonstrated continued thickening of root canal walls, root lengthening and apical closure.

**Chen YJ et al ( 2015)**[15] investigated the cytobiological effects of autologous platelet-rich fibrin (PRF) on dental pulp stem cells (DPSCs). A novel transplant consisting of cell-sheet fragments of DPSCs and PRF granules was adopted to regenerate pulp-dentin-like tissues in the root canal. PRF promoted the proliferation of DPSCs in a dose and time-dependent manner and induced the differentiation of DPSCs to odonto-/osteoblastic cells by increasing the expression of the ALP, DSPP, DMP1 and BSP genes.

**Elgendy EA et al (2015)**[23], studied the clinical and radiographical outcome of non cyrsatalline hydroxyapatite (NcHA) bone graft with or without PRF, in the treatment of intrabony periodontal defects. In split-mouth study design, patients having two almost identical intrabony defects with clinical probing depth of at least 6 mm were selected and randomly divided into two groups, In Group I, Non-crystalline hydroxyapatite (NcHA) was alone was placed and in Group II, Non-crystalline hydroxyapatite (NcHA )with PRF was used to fill the bony defect. There was a significantly greater periodontal pocket depth (PPD) reduction and clinical attachment gain (CAL) in group II (NcHA+PRF) at 6 month postoperatively.

**Bakhtiar H et al (2017)**[5] reported the clinical and radiographic results of root canal revascularization using PRF which was performed on 4 immature teeth with necrotic pulps. In initial visit, after access cavity preparation, the root canals were irrigated with 1.5% sodium hypochlorite [20 ml/canal, 5 minutes] followed by saline (20 ml/canal, 5 minutes) & triple antibiotic paste dressing was given. After 3 weeks, bleeding was evoked into root canal over which PRF clot was placed. Biodentine (Septodont, Saint-Maur, France) was placed directly over the PRF. The teeth were restored permanently with glass ionomer cement and composite resin. Clinical examinations revealed that all cases were asymptomatic at the recall appointments at 1, 3, 6, 12, and 18 months. Radiographs revealed resolution of the periapical lesions, further root development, and apical closure in all cases.

**HUMAN DENTAL PULP STEM CELLS**

**Gronthos S et al (2000)**[29] for the first time isolated and identified dental pulp stem cells (DPSCs) from extracted Human third molar using enzyme isolation method. DPSCs exhibited higher proliferative rate which maintained even in subculturing. When DPSCs were transplanted into immunocompromised mice , they generated a dentin like structure lined with human odontoblast like cells that surround a pulp like interstitial tissue with hydroxyapatite / β-tricalcium phosphate (HA/ β-TCP). In vitro DPSCs have been shown to produce sporadic but densely calcified nodules.

**Liu J et al (2005)**[53] used porcine dental pulp cells to study the effects of ethylene diamine tetraacetic acid – soluble dentin extract and mineralization supplement (ascorbic acid, β- glycerophosphate) on their differentiation and mineralization at day

14, 21 and 28 days to better understand tissue injury and repair in the tooth. The study concluded that mineralization supplement and dentin extract induces both dental pulp cell differentiation and subsequent mineralization.

**Huang G T-J (2006)**[36] reported the cell differentiation potential growth on dentin. The pulp cells after being seeded onto mechanically and chemically treated dentin surface which appeared to establish an odontoblast-like morphology with a cytoplasmic process extending into a dentinal tubule revealed by scanning electron microscopy analysis.

**Min J H et al (2011)**[61] investigated the stemness of human adult dental pulp cells, obtained from extracted third molars. The result indicated that, during early passages, human dental pulp primary cells contain a population of stem cells that showed significant potential for differentiation into multiple lineages, including dentinogenic and osteogenic features and dentinogenic potential of these cells emerged at specific passages and decreased gradually in primary culture.

**Atari et al (2011)**[4] Established a protocol that allows the reproducible isolation and identification of stem cells that we refer to as "Dental Pulp Pluripotent Stem Cells" (DPPSCs). The cells were isolated by culturing them in media containing LIF (leukemia inhibitor factor), EGF (endothelial growth factor), PDGF (platelet-derived growth factor). DPPSCs were identified as cells with the phenotypes CD13+, SSEA4+, OCT3/4+, NANOG+,SOX2+, LIN28+, CD14+, CD29+, CD105+, CD34-, CD45-, CD90-, STRO1- and CD146-.

**Jang JH et al (2016)**[40] Characterized human dental pulp cells (HDPCs) obtained by different culture methods to establish the most suitable methodology for dental tissue engineering and regenerative endodontic applications. HDPCs were isolated by the outgrowth method (HDPCs-OG), the enzymatic digestion method (HDPCs-ED), or the combination of both methods (HDPCs-Combined). The expression of mesenchymal stem cell markers (CD105, CD90, and CD73) was investigated. Differentiation markers were analyzed by quantitative reverse-transcription polymerase chain reaction (RT-PCR) and western blotting. They found that the methods for isolating and culturing HDPCs will influence the differentiation capacities of cells. HDPCs-OG and HDPCs-ED were preferably differentiated into adipogenic and osteogenic cells, respectively.

**Apel C et al (2017)**[2] compared the mineralized matrix production of human dental pulp stem cells (DPSC), cultured on 17 different well-characterized polymers. Success of differentiation was analyzed by quantitative RealTime PCR, alkaline phosphatase (ALP) activity and visualization of calcium accumulations by alizarin red staining with subsequent quantification by colorimetric method. Mineralization on poly(tetrafluoro ethylene) (PTFE), poly(dimethyl siloxane) (PDMS), Texin, poly(epsilon-caprolactone) (PCL), polyesteramide type-C (PEA-C), hyaluronic acid, and fibrin was significantly enhanced ($p<0.05$) compared to standard tissue culture polystyrene (TCPS) as control.

**GENE EXPRESSION**

Studies have shown that the dental pulp cells can proliferate and differentiate into osteoblast- like or ododntoblast – like cell. They expressed type I collagen and other non collagenous proteins including Osteocalcin (OCN), Osteonectin (ON), Osteopontin (OPN), Alkaline phosphatase (ALP), Bone sialoprotein (BSP), Dentin sialoprotein (DSP), Dentin matrix protein 1 (DMP-1) and Dentin sialophosprotein (DSPP).

**D' souza R N et al (1997)[18]** compared the in vivo temperospatial expression patterns of two dentin non collagenous protein genes , Dentin matrix protein 1 (DMP), and Dentin sialophosphoprotein (DSPP) in developing molars. The study concluded that developmental expression patterns of DMP-1 and DSPP are distinct, implying that these molecules serve different biological functions in vivo.

**Narayanan K et al (2001)[66]** investigated the biological function of dentin matrix protein 1 (DMP1), using stable transgenic cell lines and cell lines and adenovirally infected cells overexpressing DMP-1. The result demonstrated that the ability of antisense DMP-1 to inhibit induction of DSP and DMP-2 suggests that DMP-1 must acts as an important molecule during the differentiation of neural crest derived mesenchymal cells into terminally differentiated odontoblasts in vivo.

**Narayanan K et al (2006)[65]** demonstrated that Dentin matrix protein 1 (DMP-1), which is localized in the nucleus during early differentiation of odontoblasts, is able to bind specifically with DSPP promoter and activate its transcription.The DNA binding domain in DMP -1 resides between amino acids 420 and 489.

**Chaussian C et al (2009)**[14] investigated whether DMP 1 can be cleaved by MMP-2 and if cleavage products can act as signalling molecules on transformation of dental pulp cells into putative odontoblasts. The study demonstrated that MMPs, which are able to cleave DMP1 into peptides of various molecular sizes, represent good candidates for regulating the function of extracellular molecules during dentin development or during pulp repair.

**Martinez EF et al (2010)**[59] examined the immune-expression of DMP-1 in tooth germs of 7 human foetuses at different gestational ages. The study concluded that DMP-1 is present in all developing dental structures (dental lamina, enamel organ, dental papilla) presenting few immunoexpression variations, with no staining in mineralized enamel and dentin.

**Kang CM et al (2016)**[42] compared the gene expression patterns and functions in human umbilical cord (UC) and dental pulp (DP) containing mesenchymal stem cells (MSCs). UC showed higher expression of CD29, CD34, CD44, CD73, CD105, CD146, and CD166. qRT - PCR analysis showed that CD146, CD166, and MYC were expressed 18.3, 8.24, and 1.63 times more highly in UC, whereas the expression of CD34 was 2.15 times higher in DP. Immunohistochemical staining revealed significant differences in the expression of genes (DSPP, DMP1, and CALB1) related to odontogenesis and angiogenesis in DP.

**Soares DG et al (2017)**[87] evaluated the odontogenic potential of human dental pulp cells (HDPCs) cultured on calcium aluminate enriched chitosan-collagen scaffold (CHC-CA). It was found that after 14 days, cells in the CHC-CA scaffold featured the

highest deposition of mineralized matrix and expression of odontoblastic markers (ALP activity and DSPP/DMP-1 gene expression).

**DENTIN CHIPS**

**Yoshida T et al (1998)**[102] evaluated the biocompatibility of freeze-dried allogenic dentin powder and True Bone Ceramics (TBC), as apical barrier materials in dogs. In histopathology, the teeth filled with the dentin powder and those filled with TBC, multinucleate giant cells appeared on the bone resorption surface and the surface of the root canal wall, and marked bone resorption and positive tartrate-resistant acid phosphate staining were observed. Hard tissue had formed on the root canal wall and the filling material after 3 months.

**Moharamzadeh K et al (2008)**[63] conducted study on extracted bovine dentine, which was processed mechanically and chemically with inorganic and organic solvents (isopropanol), and sterilised. The in vitro biocompatibility on human gingival fibroblasts was assessed by the Alamar Blue assay and the in vivo biocompatibility evaluated by implantation of the processed dentine into rat's femur. The dentine showed excellent biocompatibility in vitro, stimulated formation of new bone and was completely incorporated into the bone in vivo.

**Ibrahim Hussain et al (2012)**[39] studied osteoconductive and osteogenic properties of processed bovine dentin using a robust rabbit calvarial defect model. White rabbits were operated to create three circular defects in the calvaria. The CT data showed the density in the area grafted with the dentin-based material was higher than the surrounding bone and the areas grafted with autologous bone, after 1week and 6

weeks of healing. Histological examination of the defects filled with the dentin product after 6 weeks showed soft tissue encapsulation around the dentin particles.

**Kim KY et al (2012)[48]** developed a novel bone grafting material that incorporates autogenous teeth (AutoBT). AutoBT contains organic and inorganic mineral components and is prepared from autogenous grafting material, thus eliminating the risk of an immune reaction that may lead to rejection. It was used at the time of implant placement, simultaneously with osteoinduction surgery, and excellent bony healing by osteoinduction and osteoconduction was confirmed.

**Saeed K W et al (2015)[80]** prepared fine dentin particles from human extracted teeth and filled in the cavity made in femur of white rabbits (experimental groups) and those cavities left unfilled served as control group. Five rabbits were sacrificed after 2, 4, and 6 weeks intervals. Tissues were processed for histological examination, which showed dentine-bone union with no signs of inflammation.

**Lymperi S et al (2015)[56]** assessed the adhesion and migration of dental stem cells on human pulp ceiling cavities made in third molars filled with MTA, Bio-Oss, and dentin chips in an experimental model, and observed with electron microscopy. The highest number of cells was recorded on dentin chips and MTA. This study highlights that MTA and dentin chips have a greater potential compared to Bio-Oss regarding the attraction of dental stem cells and are good candidates for bioengineered pulp regeneration.

**Kang KJ et al (2017)[43]** compared the efficiencies of osteogenic differentiation and in vivo bone formation of hydroxyapatite – tricalcium phosphate (HA-TCP) and

demineralized dentin matrix (DDM) on human dental pulp stem cells (hDPSCs). DDM contains inorganic components as with HA-TCP, and organic components such as collagen type-1. Due to these components, osteoinduction potential & dentinogenic gene expression of DDM on hDPSCs was remarkably higher than that of HA-TCP.

**NANO-HYDROXYAPATITE**

**Brandell W et al (1986)[10]** used Demineralized dentin, Hydroxyapatite, or Dentin chips as an apical filling material which were condensed into the apical 2mm of canals with perforated apexes in anterior teeth of adult cynomolgus monkeys. Perforated apexes were obturated 2 mm short of their radiographic apexes served as controls. The amount of hard tissue formation and the degree of inflammation were evaluated after 3 and 6 months. After 6 months the samples with apical plugs of hydroxyapatite had more hard tissue formation and less inflammation than the others.

**Schwarz F et al (2006)[82]** evaluated the healing of intrabony peri-implantitis defects following application of a nanocrystalline hydroxyapatite (nHA) or a bovine-derived xenograft in combination with a collagen membrane (Bio-Gide) (BDX+BG). At 6 months after therapy, nHA showed a reduction in the mean periodontal pocket depth (PPD) from 7.0±0.6 to 4.9±0.6 mm and a change in the mean clinical attachment loss (CAL) from 7.5±0.8 to 5.7±1.0 mm. In the (BDX+BG) group, the mean PPD was reduced from 7.1±0.8 to 4.5±0.7 mm and the mean CAL changed from 7.5±1.0 to 5.2±0.8 mm. It can be concluded that at 6 months after surgery both therapies resulted in clinically important PPD reductions and CAL gains.

**Shayegan A et al (2010)**[84] compared the pulp response of pig primary teeth after capping with Nano-hydroxyapatite (nHA) & Formocresol(FC) in pulpotomy and nHA & Calcium hydroxide $(CaOH)_2$ in direct pulp capping. Teeth of two 4-month old pigs were pulpotomized and capped with these materials. After four weeks, specimens were prepared for histological examination. In the pulpotomy groups, there was a significant difference between nHA and FC in terms of pulp response, hard tissue formation and normal pulp tissue preservation. In the direct pulp capping groups, there was no significant difference between nHA and $Ca(OH)_2$ in terms of criteria mentioned above.

**Yang X et al (2010)**[101] investigated the in vitro and in vivo behavior of dental pulp stem cells (DPSCs) seeded on electrospun poly(epsilon-caprolactone) (PCL)/gelatin scaffolds with or without the addition of nano-hydroxyapatite (nHA). For the in vitro evaluation, DNA content, alkaline phosphatase (ALP) activity and osteocalcin (OC) measurement showed that the scaffolds supported DPSC adhesion, proliferation, and odontoblastic differentiation. Moreover, the presence of nHA upregulated ALP activity and promoted OC expression. Real-time PCR data confirmed these results. SEM micrographs qualitatively confirmed the proliferation and mineralization characteristics of DPSCs on both scaffolds.

**Vahabi S et al (2012)**[96] evaluated the quality and quantity of regenerated bone using Bio-Oss, hydroxyapatite/tricalcium phosphate (HA/TCP) and mesenchymal stem cell sheet loaded HA/TCP scaffolds. Bilateral bone defects were then prepared in the jaws using trephine burs. The bilateral bone defects were created in jaws using trephine burs and randomly filled with HA/TCP, Bio-Oss, or HA/TCP + MSCs. One defect

served as a control and was left as an empty cavity. Histological and histomorphometric evaluations were conducted after 6 weeks proved MSC-loaded HA/TCP scaffold is a more effective alternative than Bio-OSS or HA/TCP in inducing bone regeneration.

**Sivashankar et al (2013)**[85] used PRF and HA to fill a large bony defect associated with upper front tooth. Radiological examination after 2 years showed the HA crystals have been completely replaced by new bone. Thus the use of PRF in conjunction with HA crystals might have accelerated the resorption of the graft crystals and would have induced the rapid rate of bone formation.

**Qi H et al (2016)**[76] investigated behaviour of poly (l-lactide) (PLLA) and poly (ε-caprolactone) (PCL) nanofibrous scaffolds containing hydroxyapatite which was added to mimic the native bone extracellular matrix. Cell count kit-8 assay, alkaline phosphatase (ALP) assay, and osteocalcin assay were applied to observe the MC3T3-E1 cells proliferation, differentiation on the composite scaffolds. The level of MC3T3-E1 differentiation was evaluated through the ALP activity and osteocalcin concentration, which showed higher value with HAP containing (PLLA/PCL/HAP) than that ones without.

**RADIOPACITY**

**Tanomaru-filho M et al (2008)**[93] evaluated the radiopacity of 5 root-end filling materials (white MTA-Angelus, grey MTA-Angelus, IRM, Super EBA and Sealer 26). Five specimens (10 mm diameter X 1 mm thickness) were made from each material and radiographed next to an aluminum stepwedge. Radiodensity was measured using VIXWIN 2000 software and converted into millimeters of aluminum

(mm Al). The tested root-end filling materials presented different radiopacities, white/grey MTA and Super EBA being the least radiopaque materials

**Oporto V et al (2014)[68]** analysed bone formation clinically and radiographically by the use of coagulum , freeze-dried bone allograft (FDBA), autologous bone, and a combination of autologous bone with FDBA in artificially created defects in the parietal bone of rabbits. The occlusal film radiography was taken and bone density was measured by a software. Autologous bone showed the best behavior, clinically as well as radiographically. However, FDBA is a good option as an alternative to autologous bone, as its behavior was slightly lower over time.

**Bucchi C et al (2017)[11]** conducted a study to evaluate the radiopacity of three different synthetic bone grafts in rabbit calvaria, over 3 months, using cone beam computed tomography (CBCT). Four critical-size defects were made on the calvaria rabbits and classified into three groups based on which allograft material used to fill: Osteon 70/30, Osteon collagen, and Osteon II groups. The fourth group received blood clot, and served as a control. The bone samples were collected and analyzed with CBCT after the 1st, 2nd, and 3rd month. After the 3rd month, the lesions with Osteon 70/30 graft showed the highest radiopacity values, followed by Osteon collagen and Osteon II groups.

## MATERIALS AND METHODOLOGY

This study was conducted with approval from Institutional ethical committee Tamil Nadu Govt. Dental College, Chennai for extraction of Human third molar teeth (for isolation of pulp tissue for cell culture and dentin chips preparation) and maxillary canine teeth (for Dentin disc preparation).

### MATERIALS

a) **For PRF Preparation**

10 ml IV blood

b) **Synthetic Radiopacifier used**

Nano-hydroxyapatite (***G Bone, SHAG31- Surgiwear company***) (Fig. 2)

c) **Chemicals For Isolation Of Pulp Tissue , Cell Culture, Cell Viability, MTT Assay, Mineralization Assay, Western Blot**

Dulbecco's Modified Eagle's medium(DMEM) (***GIBCO***) (Fig. 5a), Fetal bovine serum (FBS) (***GIBCO***) (Fig.5b), Saline Trypsin Versene (STV) (***GIBCO***) (Fig.7), Collagenase type I (***GIBCO***) (Fig. 6), F-12 (IX) Nutrient mixture (HAM) (***GIBCO***), Antibiotic mixture (***GIBCO***) (Fig.6), EDTA, Povidone-iodine solution, Dimethyl sulfoxide (DMSO)(***SIGMA-ALDRICH***) (Fig.8), Phosphate buffered saline (PBS), MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Fig.9), 4mM HCl, 0.1% NP40 in isopropanol, 4% Formaldehyde, 100mM Alizarin red S (ARS) (Fig. 10), Deionized $H_2O$ ($diH_2O$), 10% Acetic acid, 10% Ammonium hydroxide ($NH_4OH$) , IGEPAL, Sodium chloride (NaCl), Tris, Phenylmethylsulphonyl fluoride (PMSF), Aprotinin, Leupeptin ,Methanol, Phosphoric acid ($H_3PO_4$) , Bovine serum albumin (BSA), Acrylamide, N,N'-Methylenebisacrylamide,

Tetramethylethylenediamine (TEMED), Ammonium per sulphate (APS), Tris HCl, Glycine, Sodium dodecyl sulfate (SDS**),** 2-mercaptoethanol, Sucrose, Bromophenol blue, Sodium chloride (NaCl), Magnesium chloride ($MgCl_2$), Glycerol, Nitroblue tetrazolium (NBT) & 5 Bromo-4-chloro-3-indolyl phosphate (BCIP) (Fig. 13) , Anti-DMP1 (Dentin Matrix Protein-1), Anti-DSPP (Dentin Sialo-phosphoprotein), Anti-STRO1, Anti-IL-6 (Interleukin-6) (Fig.11), Anti-IL-8 (Interleukin-8) (Fig.11), Secondary antibody conjugated with HRP (Horse Radish Peroxidase enzyme) (Fig.12)

**ARMAMENTARIUM**

**a) For Preparing PRF**

Tourniquet, 10ml syringe , 10ml Test tube , Centrifugal machine ( *REMI C 854/6*) (Fig.20)

**b) For Dentin Chips Preparation (Fig.15)**

Diamond coated disc, Straight handpiece (NSK), Glass slab

**c) For Dentin Disc Preparation (Fig.15)**

Diamond coated disc, Straight hand piece (NSK)

**d) For Checking Radiopacity**

Digital weighing machine (Fig.15), Watch glass, Dappen dish, Mixing spatula, Teflon ring (6mm internal diameter and 8mm external diameter, 2mm height). DIGORA for windows 2.9.113.490 (***SORDEX)*** (Fig.16**)**, DIGORA sensor plate (31mm× 41mm), X-ray machine, 8mm Aluminium step wedges ( ***NDE Flaw Technologies Pvt Ltd, Chennai)*** (Fig.14)

d) **For Enzymatic Isolation And Cell Culture**

Straight hand piece (NSK Slow Speed Handpiece) ,Barbed Broaches (#20), 100 mm Petridish, Diamond disc, Mandrel, Cool Packs, No: 15 Blade, 15ml and 45ml Conical Centrifuge tubes (Fig.18c) , Micropipettes (Fig.18c), Centrifuge, Vortexer (***REMI CM 101***) (Fig.19), Millipore Filter (0.22 µm) (Fig.17a), 70µm Cell strainer (Fig.17b) , T25 Flask (Fig.17c) , Inverted Phase Contrast Microscope (***OPTIKA XDS-3***) (Fig.23), Cell Culture $CO_2$ Incubator (***LEEC Research*** ) (Fig.22) , Laminar air flow chamber (Fig.21), Cover Slip, Neubauer haemocytometer.

e) **MTT Assay And Mineralization Assay**

Multimode microplate reader (***Molecular Devices***) (Fig.26) , 96 well culture plate, 12 well culture plate (Fig.18a)

f) **ELISA and Western Blot Assay**

ELISA plate (Fig.18b) ,6 well culture plate, Cell scraper, Microcentrifuge Tubes, Nitrocellulose Membrane and Whatman Filter Papers of 8 X 6 cm Size, Measuring cylinder, Glass plates , spacer, Well comb (Fig.24) , Gel cassette holder (Fig.25), Vertical electrophoresis unit (***GE Health Care***) (Fig.26), Multimode microplate reader (***Molecular Devices***) (Fig.27)

I. **FOR MEASURING ALUMINIUM EQUIVALENT OF RADIOPACITY**

a. **Preparation of materials**

**Human blood collection and preparation of Platelet rich fibrin (PRF)[20]**

Human whole blood samples from healthy volunteers were collected in 10 ml glass test tubes without anticoagulant and immediately centrifuged at 3000 rpm for 10

minutes at room temperature (23°C). After the centrifugation process, the platelet-rich fibrin clot formed in between the top most serum layer (platelet poor plasma ) and bottom most red blood cell layer was carefully isolated (Fig 1).

- **Nanohydroxyapatite (nHA)**

Commercially available synthetic granular Nano-hydroxyapatite is ground to powder and stored in eppendorf tubes .(Fig 2b)

- **Dentin chips (DC)[56]**

Freshly extracted third molars were collected from individuals aged 20–30years & kept in 0.5% sodium hypochlorite to remove soft tissue and prevent bacterial growth. Before further preparation, the teeth were rinsed and autoclaved (121°C, 15 min). Dentin chips were prepared from the root portion of the tooth using an autoclaved diamond coated disc in a low- speed handpiece without water spray and collected on an autoclaved glass slide. (Fig 3a) Finally stored in an eppendorf tubes. (Fig 3b)

- **Preparation of dentin disc[37]**

The dentin discs were obtained by cutting the roots of non-carious freshly extracted human maxillary canine teeth in 1mm thick sections with a water-cooled carborundum disk. (Fig.4)

**a. Preparation of samples**

**Incorporation of bioactive agents into PRF**

The PRF obtained is weighed using digital weighing machine. (Fig.28) Into the weighed PRF, 50 wt % of nanohydroxyapatite and 50 wt% of dentin chips were mixed separately. Fig.29a) The sample is standardized using Teflon rings. (Fig 29b)

Group 1- Control- Dentin discs (n=3)

Group 2 - PRF (n=5)

Group 3 - PRF modified with 50wt% nanohydroxyapatite (nHA) (n=5)

Group 4 - PRF modified with 50wt% dentin chips (DC) (n=5)

**Procedure** [37]

The samples were digitally radiographed together with 8mm aluminium step wedge using DIGORA Software for Windows 2.9.113.490. (Fig.30) Five readings of density values were obtained from each sample (Fig.31a) and the arithmetic mean of these repetitions were calculated. The dental x-ray unit was set at 50 Kvp, 10mA, and exposure time of 0.3 sec, with a focus-film distance of 30 cm. The density of stepwedge steps was measured and a graph of radiographic density versus the thickness of aluminium was constructed. The aluminium equivalency values for the mean radiographic density readings of each sample was calculated using conversion formula

$$\frac{A \times 1}{B} + \text{mmAI immediately below RDM}$$

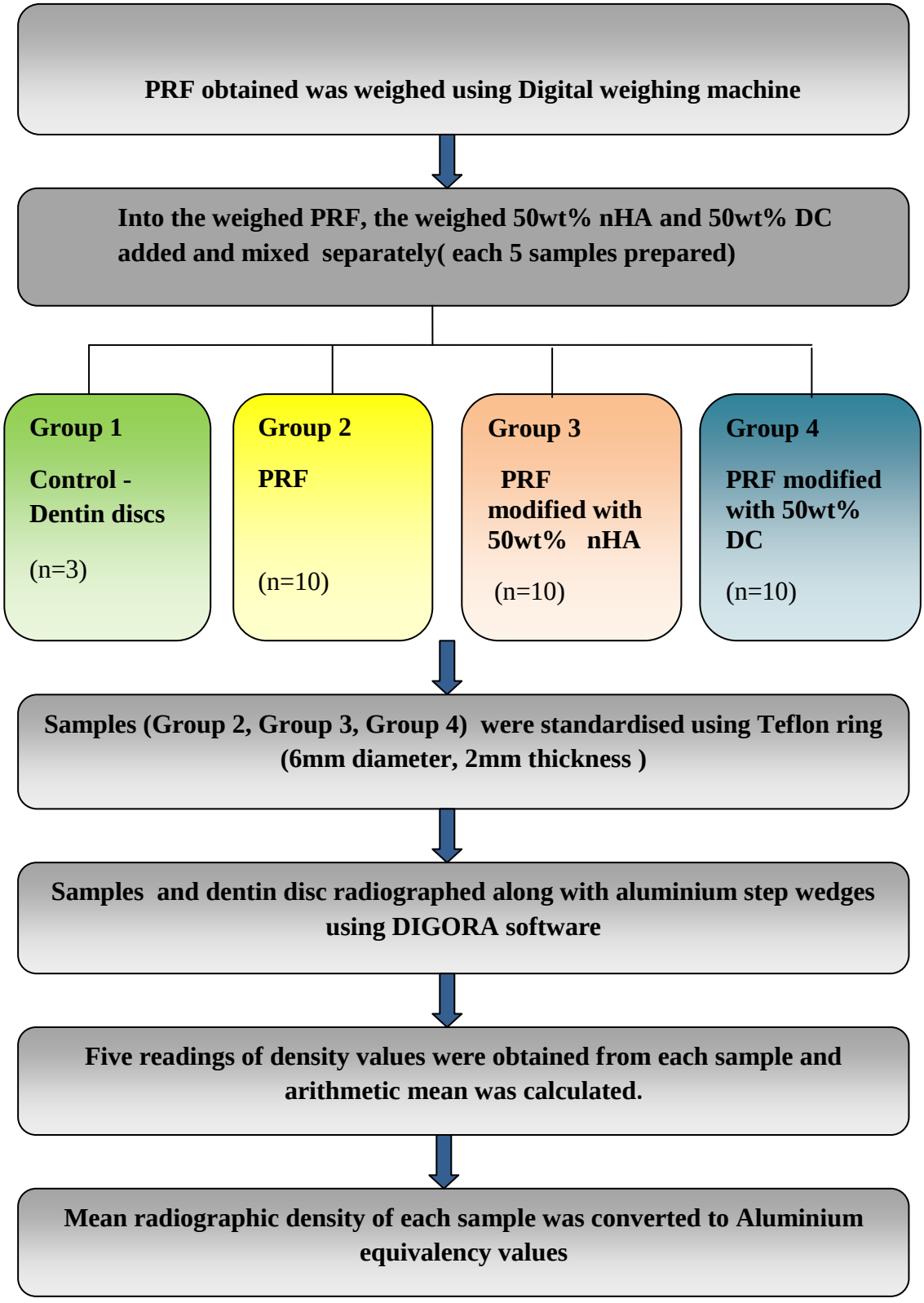
where ,

**A** = radiographic density of the material (RDM) – radiographic density of the aluminium stepwedge increment immediately below RDM

**B** = radiographic density of the aluminium stepwedge increment immediately above RDM – radiographic density of the aluminium stepwedge increment immediately below RDM,

**1** = 1mm increment of the aluminium stepwedge.

**PROCEDURAL STEPS FOR CHECKING RADIOPACITY**



## I. FOR CELL CULTURE BASED ASSAYS

### a) Preparation of material

- **Platelet rich fibrin extract ( PRFe)[47]**

The platelet-rich fibrin (PRF) was then frozen at 80°C for 1 hour to separate the trapped growth factors and cytokines from the fibrin mesh. The separated liquid (extract) was harvested without the fibrin fibers by means of centrifugation at 1200rpm for 10 minutes and constituted the PRFe.

- **Nanohydroxyapatite extract and Dentin chips extract [89]**

The extracts of nano-hydroxyapatite and dentin chips were made in separate falcon tubes using DMEM media. The tubes were incubated at 37 °C for 24 h. The obtained extracts were sterilized using 0.22 μm filter, followed by 1% antibiotic-antimycotic & 10% fetal bovine serum (FBS) were added to all of them.

### b) Preparation of samples

From 1mg/ml stock solutions of PRF extract, Nano-hydroxyapatite (nHA) and Dentin chips (DC ) extracts , further concentrations (100μg/ml, 10μg/ml, 1μg/ml, 100ng/ml, 10 ng/ml,1ng /ml) were prepared by serial dilution.

Group A- DMSO (Control)

Group B- PRF

Group C– PRF+nHA

Group D– PRF+DC

## i. CELL CULTURE

**Reagents:**

1. **Dulbecco's Modified Eagles Medium (DMEM)** (Fig.5a)

   The powdered media was dissolved in 900 ml of autoclaved distilled water in a conical flask under sterile conditions. Sodium bicarbonate of 3.7 g was added and stirred until it completely dissolved and the pH of the medium was adjusted to 7.2 by adding 3 ml of 1N HCl. Following this antibiotics 10ml penicillin-streptomycin (10,000 units/ml of Penicillin G sodium and 10,000 µg/ml of streptomycin sulphate) solution and 1ml amphotericin B (250 µg/ml of amphotericin B fungicide) solution were added. The final volume was made upto 1litre with distilled water and sterile filtered with Millipore filter (0.22 µm pore size).The medium was then dispensed into sterile container and stored at 4ºC.

2. **DMEM with 10% Fetal bovine serum (FBS)**

   10ml of FBS was made upto 100ml using sterile DMEM and was stored in sterile container in aseptic condition at 4°C.

3. **Phosphate buffered saline (PBS )**

4. **Collagen type I** (Fig.6)

5. **Saline Trypsin Versene (STV)** (Fig.7)

   10X Saline is prepared using 8 g NaCl, 0.4 g KCl, 1.0g D-Glucose and 0.35g $NaHCO_3$ dissolved in 100 ml water. 10X saline was filter sterilized and stored at 4ºC.For preparation of Versene, 1g of EDTA was weighed and added to 90ml of distilled water. The solution was completely dissolved by adding 5N NaOH in drops thereafter filter sterilized and stored at 4ºC. For STV

preparation, 100mg of trypsin, 10ml of 10X saline and 2.5ml of versene were added and was made upto 100ml using double distilled water. It was then filtered, sterilized and stored at 4ºC.

6. **Osteogenic medium (OM )**

   The DMEM supplemented with 100nM dexamethasone, 10mM β-glycerophosphate and 50µg/ ml ascorbic acid.

**Procedure:**

- **Sample collection**

20 Freshly extracted impacted third molars were collected from Department of oral surgery between age group of 18-25 years. The teeth were washed in povidone-iodine solution initially followed by rinse with phosphate buffered saline containing antibiotics (100 IU/ml penicillin and 100 µg/ml streptomycin). A horizontal section was made along the cervical line of collected third molars to split the teeth with diamond disc on straight handpiece. The pulp tissue was collected with sterile broaches (#20) (Fig.32) and pulp tissue was transported to laboratory (***Apex Biotechnology Training and Research Institute, Guindy, Chennai*** ) in a 15ml conical tube containing DMEM with 20% FBS placed in cool pack. (Fig.33)

- **Enzymatic isolation of Human dental pulp cells (hDPSCs)**

The human dental pulp tissues were collected and minced with scalpel (No: 15 blade) in 100mm petridish in DMEM (Fig.34) followed by pelleting of minced samples using centrifugation at 1200rpm for 10 minutes. (Fig.35, 36) The pelleted tissues were digested in a solution 3mg/ml collagenase type I containing medium for 60 minutes at 37°C (Fig37). The suspended cells were centrifuged at 1200 rpm for 10

min. The solution thus obtained was filtered following digestion on a 70µm cell strainer and the cells (pellet) collected by centrifugation was washed twice in culture medium. The obtained single cell suspension were then resuspended into the 6ml of DMEM supplemeted with 10% FBS Serum, 2 mM L-glutamine, 100 IU/ml penicillin (Fig.38) and 100 µg/ ml streptomycin and transferred into T25 flasks. (Fig.39) The flasks were incubated in 5% $CO_2$ at 37 °C. The hDPC were cultured for 2-3 weeks. (Fig.40)

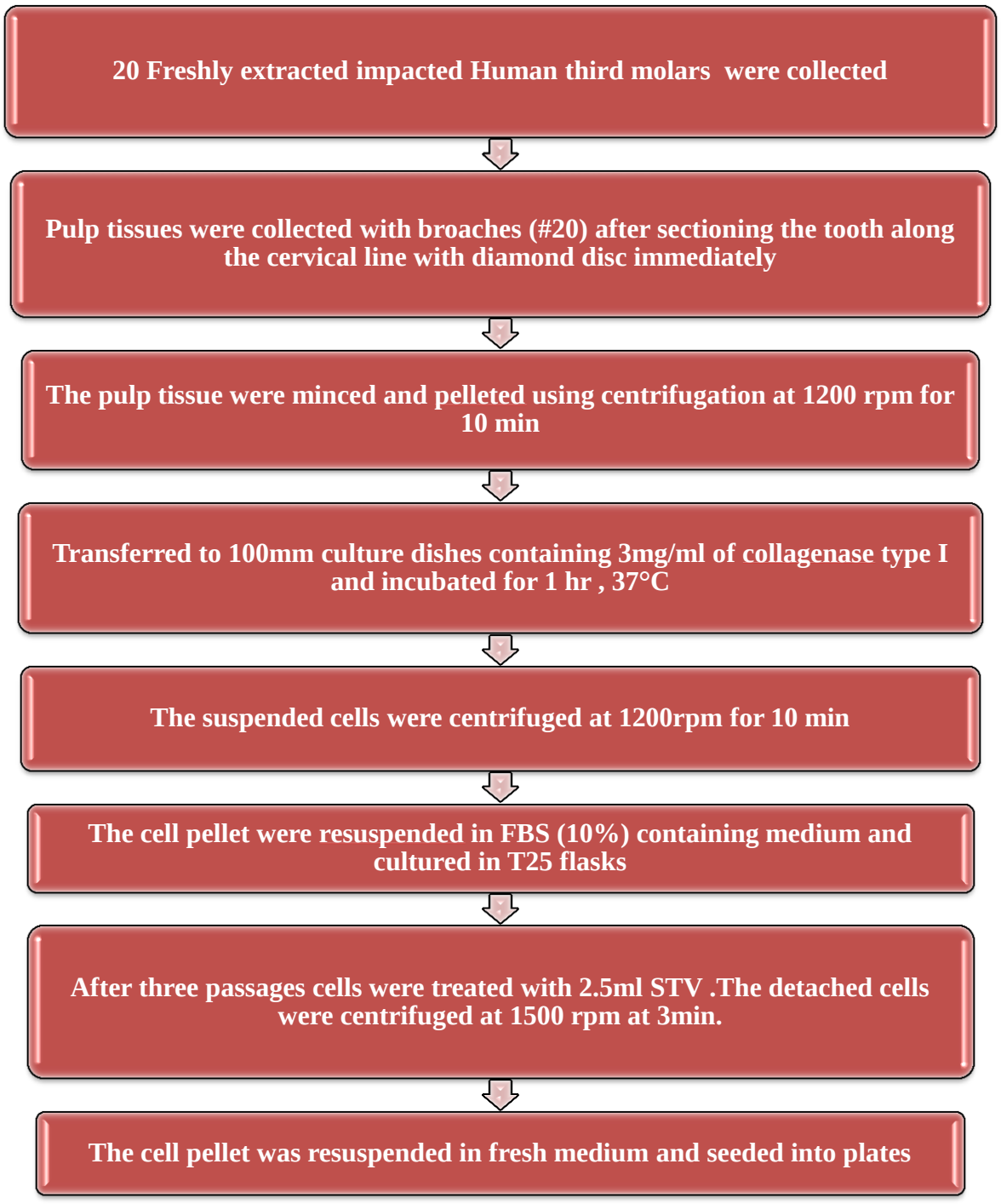
- **Passaging of cell**

The culture medium was aspirated completely and the traces of medium were removed by washing with STV. After which 2.5ml STV was added to the flask and incubated at 37ºC for few minutes until the cells start detaching from the surface. After complete detachment, STV action was neutralized using DMEM containing serum (10ml of 10% FBS –DMEM). The cells were pelleted by centrifugation at 1500rpm for 3min and the supernatant was discarded. The cell pellet was resuspended in fresh medium and seeded into flask or plates according to the requirement and incubated at 37ºC. From cell suspension a drop was placed to edge of cover slip of Neubauer haemocytometer and drop was allowed to run under cover slip by capillary action. Then cells were seen under microscope .(Fig.41-44) The number of cells was calculate using formula:

No: of cells= No. Of cells counted ×50, 000

= X cells/ ml

**PROCEDURAL FLOW CHART FOR ENZYMATIC ISOLATION OF HUMAN DENTAL PULP CELLS**



### i. CYTOTOXICITY ASSAY USING MTT

**Reagents:** MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]

**Prepare MTT solution**

MTT powder was prepared into solution using a 5 mg/ml PBS which is then mixed by vortexing or sonication. The sterilized solution was filtered and stored at -20°C.

**Prepare MTT solvent**

4 mM HCl, 0.1% NP40 in isopropanol

**Principle :** MTT assay is a colorimetric method used to measure cell viability. Yellow MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide], a tetrazole is reduced to purple formazan in the mitochondria of living cells. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related.

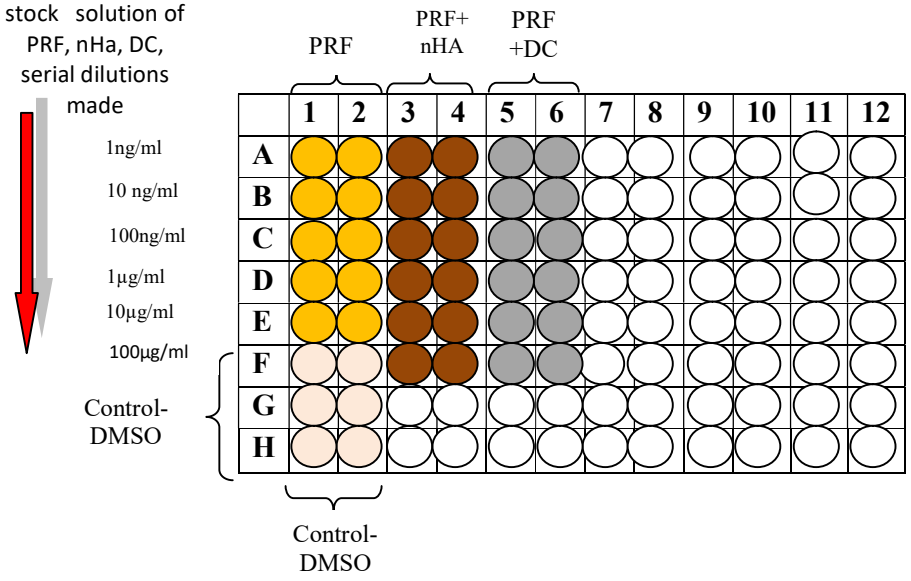
**Procedure:** The cells were seeded in a 96-well plates at the density of 5 x $10^3$ cells / well. (Fig.45) The cells were incubated in a $CO_2$ incubator at 37°C and 5% $CO_2$ under controlled humidified atmosphere overnight to allow them to attach to the plate. Discard media from cell cultures. For adherent cells, carefully aspirate the media. For suspension cells, spin the 96 wellplate at 1000 rpm, 4°C for 5 minutes in a microplate-compatible centrifuge and carefully aspirate the media. Then the cells were exposed to different concentrations (1ng/ml, 10 ng/ml, 100ng/ml, 1µg/ml, 10µg/ml 100 µg/ml) of Group B (no:1 &2 wells) , Group C (no: 3& 4 wells), Group D (no:5 & 6wells) for 48h. The DMSO (Group A) was kept as control. At the end of 48 h, 200 µl of serum-free media and 10 µl of MTT solution were added into each well & further incubated for 37°C for 3hours. At the end of incubation period, the contents of the plates were discarded by simple decantation and the plates were dried overnight at

room temperature. The purple-coloured formazan crystals formed were dissolved in 150 µl of MTT solvent by shaking at 400rpm for 15 min at room temperature in a thermo shaker. (Fig.46) Occasionally, pipetting of the liquid may be required to fully dissolve the MTT formazan. The intensity of the colour developed was absorbed at 570nm in a multimode microplate reader. The amount of absorbance is proportional to cell number. The percentage growth / viability of cells was calculated using the following formula

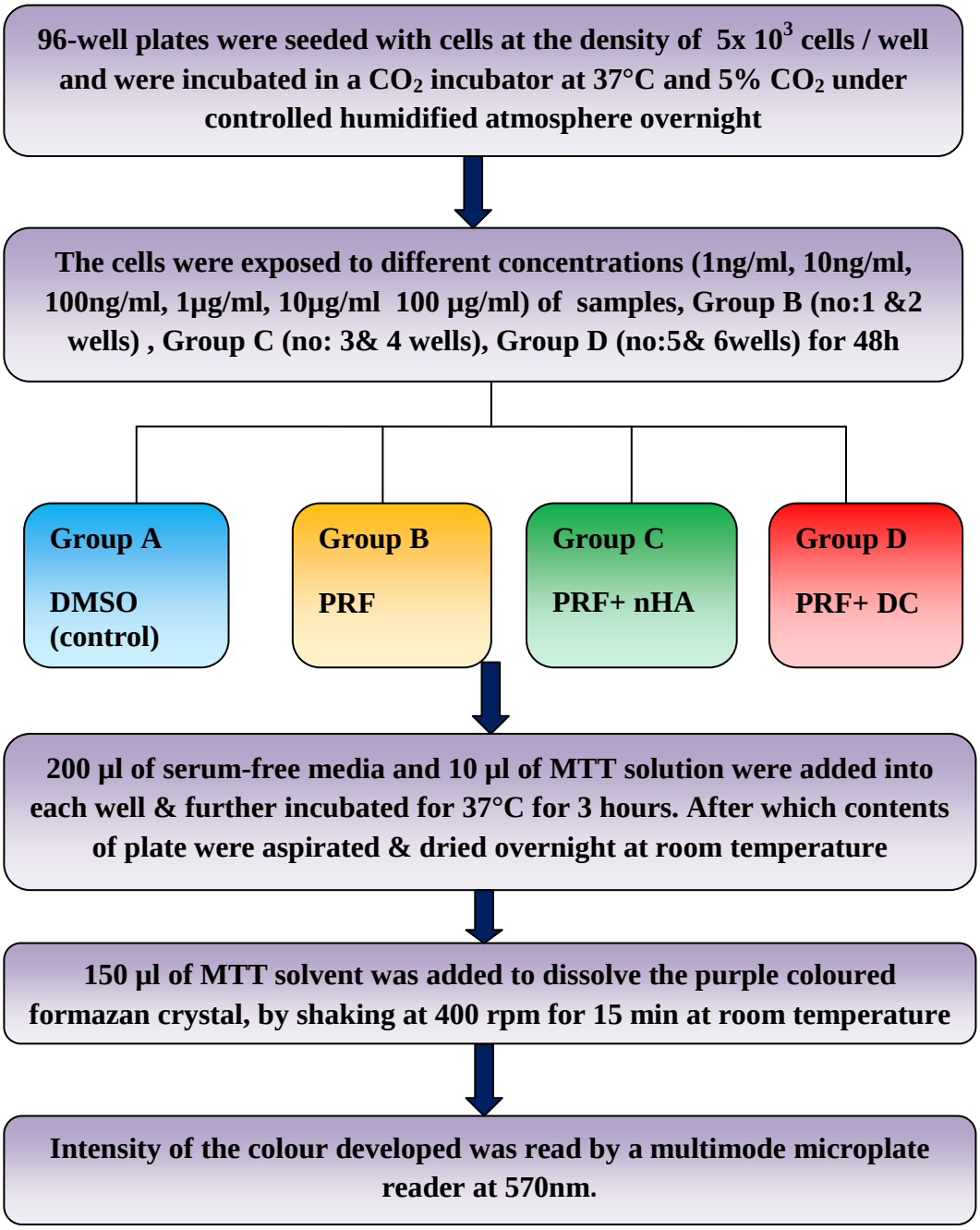
$$\% \text{ of cytotoxicity} = \frac{\text{OD control- OD test}}{\text{OD control}} \times 100$$

$$\text{Viable cells\%} = \frac{\text{OD of test}}{\text{OD of control}} \times 100$$

**Schematic representation of MTT assay**



**PROCEDURAL FLOW CHART FOR MTT ASSAY**



### ii. MINERALIZATION ASSAY

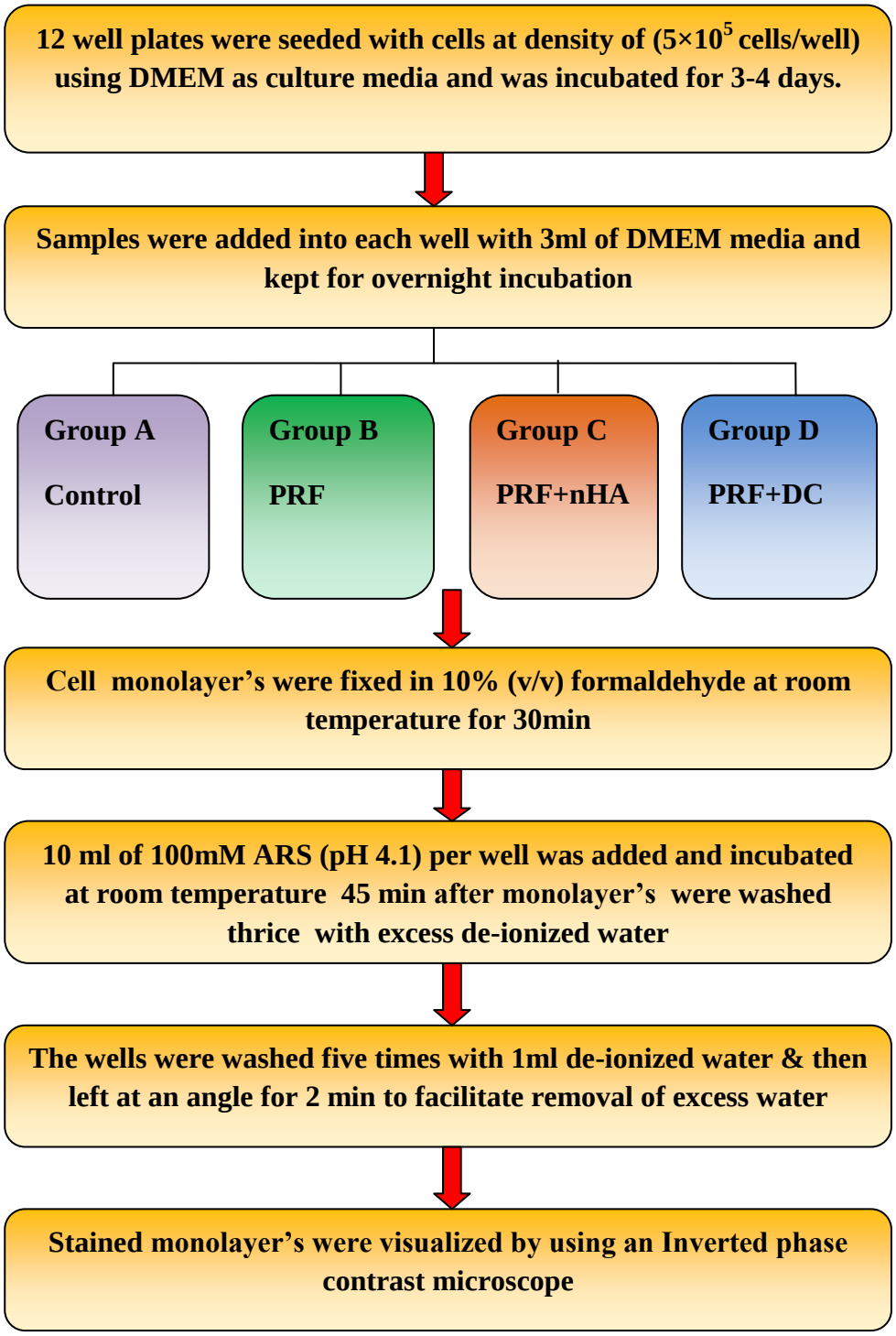
**Reagents:** DMEM media, 10% formaldehyde solution, De-ionized $H_2O$ ($diH_2O$), 100 mM Alizarin Red S, 0.10% Ammonium hydroxide ($NH_4OH$)**.**

**Preparation of Alizarin Red S:** 2g of Alizarin Red S was dissolved in 100 ml distilled water, which is mixed, and pH was adjusted to 4.1 - 4.3 with 0.1% $NH_4OH$. The dark-brown solution obtained was filtered and stored it in the dark

**Principle:** Alizarin red is an indicator of calcium phosphate deposition appearing when the matrix produced by osteoblasts mineralizes. One mole of alizarin red has capacity to bind with two moles of calcium. The binding of alizarin red gives a red colour when it binds to mineralized matrix.

**Procedure:** The cells were seeded into 12 well plates at density of $5\times10^5$ cells/well using DMEM as culture media and was incubated for 3-4 days. After the cells reach 70- 80% confluency, media was aspirated and was washed thrice with 500µl DMEM media. The samples were added into each well with 3ml of DMEM media and kept for overnight incubation. 1µg of sample dissolved in 0.5% DMSO served as control. Then the cells were fixed in 10% (v/v) formaldehyde at room temperature for 30min. The monolayers were then washed thrice with excess de-ionozed water prior to addition of 10 ml of 100mM ARS (pH 4.1) per well. (Fig.48) The plates were incubated at room temperature for 45 min with gentle shaking. After aspiration of the unincorporated dye, the wells were washed five times with 1ml de-ionized water while shaking for 5 min. The plates were then left at an angle for 2 min to facilitate removal of excess water. Stained monolayers were visualized by using an Inverted phase contrast microscope, which will be seen as bright orange-red deposits of mineralization.

**PROCEDURAL FLOW CHART FOR MINERALIZATION ASSAY**



### iii. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

**ELISA** (Enzyme-linked Immunosorbent Assay) is a plate-based assay technique designed for detecting and quantifying peptides, proteins, antibodies and hormones. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme which reacts with a colorless substrate to generate a colored reaction product.

**Reagents:**

- Assay diluent- Add 50 ml FBS in 450 ml PBS for 10% v/v FBS which is filter sterilized and stored at 4°C.
- Capture antibodies (IL-6 and IL-8 Ab), Cytokine of interest for standard (IL-6 and IL-8), Anti-mouse conjugated to horseradish peroxidase (HRP), TMB/$H_2O_2$ (Substrate solution), 2N $H_2SO_4$ (Stopping solution)

**Procedure:**

- **Preparation of standard cytokine of interest** (IL-6 and IL-8)

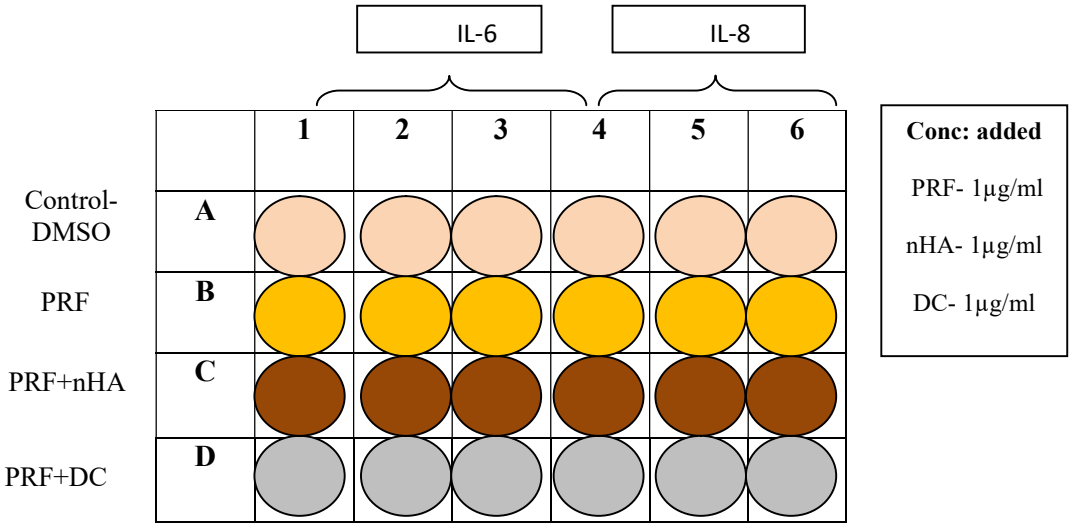
The standard was reconsitiuted with 1.0 ml of standard diluents, kept for 10 minutes at room temperature & shaked gently. The concentration of standard in stock solution is 200 pg/ml which was further diluted to the stock solution to 100pg/ml which served as highest standard before the next transfer. Further 7 points of diluted standard was set up such as 100pg/ml, 50pg/ml, 25pg/ml, 12.5pg/ml, 6.25pg/ml, 3.12pg/ml, 1.56 pg/ml, 0 pg/ml (served as blank).

- **Detection of capture antibodies – IL-6 & IL-8**

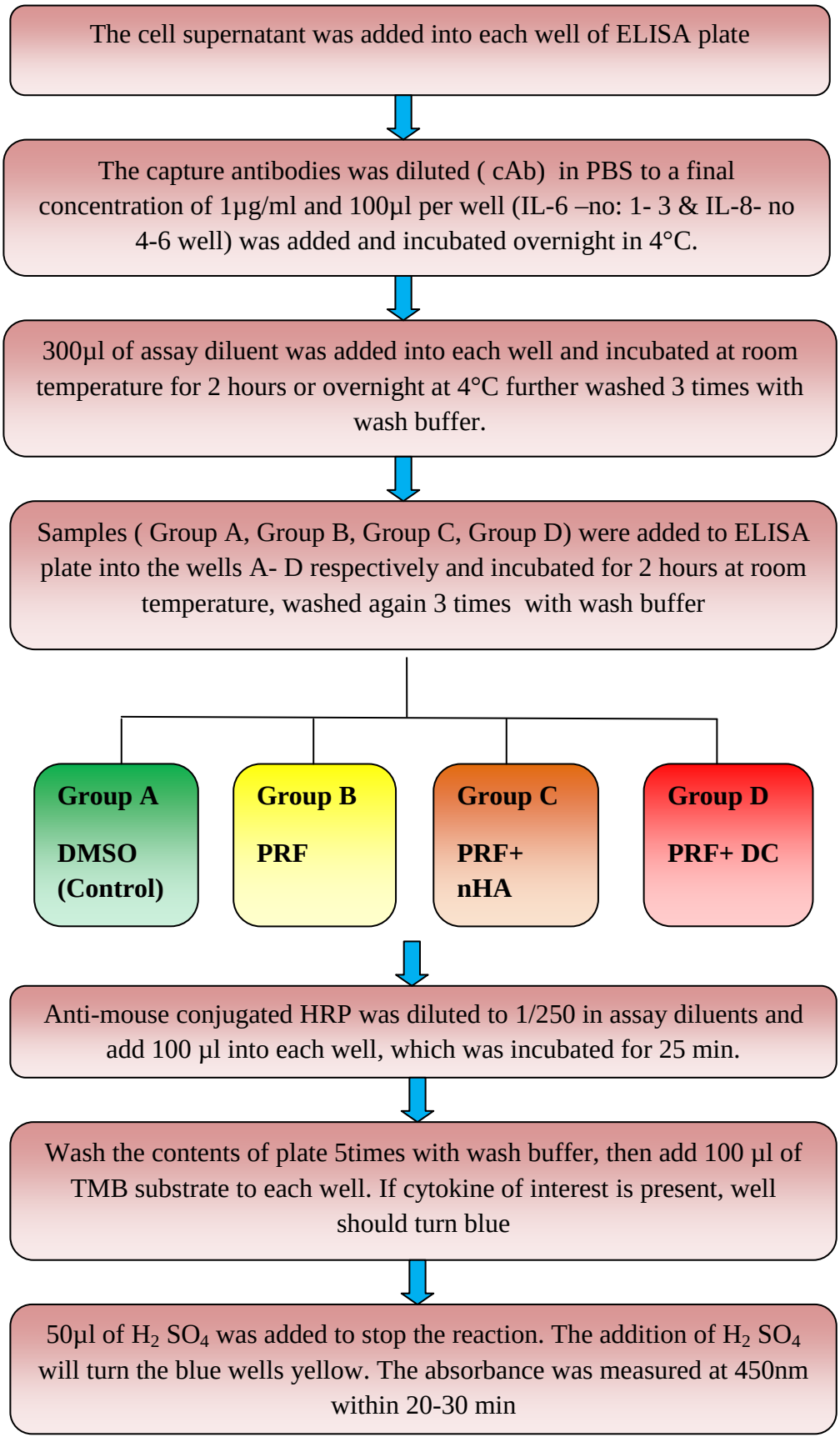
The cell supernatant was added into each well of ELISA plate. The capture antibodies was diluted (c-Ab) in PBS to a final concentration of 1µg/ml and 100µl

per well was added (IL-6 –no: 1- 3 & IL-8- no: 4-6 wells) and incubated overnight in 4°C. After that the contents from ELISA plate was disposed and dried. Then 300µl of assay diluent was added into each well and incubated at room temperature for 2 hours or overnight at 4°C further washed 3 times with wash buffer. Then the samples ( Group A, Group B, Group C, Group D) were added to ELISA plate and incubated for 2 hours at room temperature, washed again 3 times with wash buffer. Anti-mouse conjugated HRP was diluted to 1/250 in assay diluents and add 100 µl into each well, which was incubated for 25 min. Meanwhile, the TMB substrate was warmed to the room temperature. The contents of plate washed 5times with wash buffer & 100 µl of TMB substrate was added into each well. The well contained the cytokine of interest turned into blue colour. The change was monitored in standard curve; when standard curve was turned into nice gradient, 50µl of $H_2SO_4$ was added to stop the reaction. The addition of $H_2SO_4$ will turn the blue wells yellow. (Fig.47) The absorbance was measured at 450nm within 20-30 min.

**Schematic diagram representing ELISA**



**PROCEDURAL FLOW CHART FOR ELISA**



iv. **WESTERN BLOT ANALYSIS**

Western blotting (also known as protein blotting or immunoblotting) is a rapid and sensitive assay for detective and characterization of proteins. It exploits the inherent specificity by polyclonal or monoclonal antibodies. It is an analytical method wherein a protein sample is electrophoresed on an SDS-PAGE and electrotransferred onto nitrocellulose membrane. The transferred protein is detected using specific primary antibody and secondary enzyme labelled antibody and substrate.

**Buffers, reagents and stains used**

**Lysis buffer:** 1% IGEPAL, 150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, 1mM phenylmethyl sulphonyl fluoride (PMSF), 21 mg/ml Aprotinin, 10 mg/ml Leupeptin

- **Sample Loading buffer:** 20% SDS -500mM, Tris (pH 7.6), 1% Bromophenol Blue , 50% Glycerol ,1M DTT
- **Running / electrophoresis buffer:** 0.25 Tris HCl , 0.25M glycine , 0.1% SDS ( pH 8.3)
- **Transfer buffer:** 25 mM Tris , 192 mM Glycine, 20 % Methanol, 0.1 % SDS
- **Phosphobuffer saline PBS** (pH 7.2): NaCl 80 g/l , KCl 2 g/l , $KH_2PO_4$ 2.4 g/l , $Na_2HPO_4$ 4.4 g/l , 0.1 % Tween 20 (only for PBS Tween preparation)
- **Substrate buffer (for 25 ml)**: 1M Tris (pH 9.5, 2.5 ml), 4 M NaCl (0.625 ml), 1 M $MgCl_2$ (0.125 ml)
- **Development Buffer**:NBT (Nitro Blue Tetrazolium ): 0.5 g in 10 ml of 70 % Dimethyl formamide (DMF) ,BCIP (5 Bromo-4-Chloro-3-Indolyl Phosphate): 0.5 g in 7 ml of 10 % DMF

**For protein estimation**

- **Preparation of Bradfords reagent:**

  20 mg of Coomassie brilliant blue G-250 + 10 ml of methanol + 20 ml $H_3PO_4$. The final volume was made up to 200 ml using distilled water and filtered. This solution appears pale-straw in colour and was stored in the dark.

**Reagents for preparation of SDS-PAGE gel**

- **Monomer solution**: 29% Acrylamide + 1% N,N'-Methylene bisacrylamide ~30% acrylamide
- **Polymerisation enhancer**: Ammonium per sulphate (APS) : 140 mg/ml
- **Monomer and polymerisation inducer**: N,N,N', N'- Tertramethylenediamine (TEMED)

**Reagents and gel composition for SDS-PAGE**

| **Reagents used** | **Separating Gel (10%)** | **Stacking Gel (5%)** |
|---|---|---|
| Double distilled water | 4ml | 2.7ml |
| 30% Acrylamide | 3.3ml | 0.67 ml |
| Tris | 2.5ml (1.5M, pH 8.3) | 0.5ml (1M, pH 6.8) |
| 10% SDS | 0.1ml | 0.04ml |
| APS | 0.004ml | 0.004ml |
| TEMED | 0.004ml | 0.004ml |

**Procedure:**

1. **Preparation of Cell Lysate**

The cells which was exposed to samples in 6 well culture plate (Fig.49) was washed by adding cold phosphate buffered saline (PBS) and rocking gently. Then cells were scraped using PBS (Fig.50) and were pelleted at 5000 rpm for 5 min at 4ºC in a

microcentrifuge tubes. The cells were re-suspended in 50 µl of lysis buffer (Fig.51) and incubated for 45 min at 4 ºC. Subsequently, the samples were centrifuged at 1400 rpm at 4ºC for 10 min and supernatants were collected.

**2. Protein Estimation of the Obtained Cell Lysate**

A portion of cell lysate (5µl) was taken for protein content estimation which was done by using Bradford's method. The standard, bovine serum albumin (BSA) was taken at concentrations of 20 µg, 40 µg, 60 µg, 80 µg and 100 µg (i.e from 1 mg/ml stock of BSA, 20, 40, 60, 80 and 100 µl). 5µl of cell lysate samples were added to each well and the volume was made upto 200µl with 0.15M NaCl. 200µl of NaCl served as blank. The samples were incubated with 1ml of Bradford's reagent for 5 min in dark. The absorbance was read at 595nm. The readings from protein standards were used to plot a standard graph and to estimate value of the cell lysate samples. The protein concentration estimated for each group as follows: Group A (control) - 0.56mg/ml, Group B (PRF) - 0.56mg/ml, Group C (PRF+nHA)- 0.51mg/ml, Group D (PRF+DC)- 0.37 mg/ml. Equal quantities of protein samples were loaded for carrying out western blot analysis.

**3. Sample preparation**

Another portion of sample (45µl) was dissolved in 20µl sample loading buffer (Fig.52). From that 10µg/ml stock solution of each samples were made and were boiled for at least 95°C for 10-20 min.

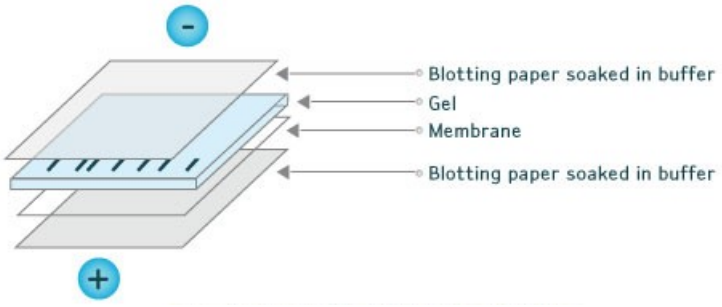
**4. SDS (Sodium Dodecyl Sulphate) Polyacrylamide Gel Electrophoresis**

For each SDS-PAGE gel, one small and one large glass plate were sandwiched which was made to slide into a holder without tightening the screws. Holder along with glass

plates were placed into a pouring stand, where glass plates are pushed all the way to the bottom before tightening the screws. A comb was placed on the top and a line was marked at 2-3cm from the bottom of the comb and then comb was removed. Separating gel was poured upto the mark and on top layer of water-saturated n-butanol was added to prevent air entrapment. The gel was allowed to polymerise for 10-15 minutes. The n- butanol was removed by inverting the gel and residual liquid was wicked off by filter paper. Then stacking gel was used to fill the remaining space between the glass plates and comb was placed. After solidification of the gel was completed, the holder with this gel was transferred to running tank for electrophoresis. The tank was filled with running Buffer (keep inside and outside buffer chambers separated). 10µl samples (control, PRF, PRF+HA, PRF+DC) were loaded into the well.(Fig.53) The electrophoresis was made to run at 50- 150 mA for 3-4 hours until the dye front reaches the bottom of the gel. (Fig.54)

**5. Western Blot**

The gel while still attached to glass plate, was transferred to box containing transfer buffer and using spatula gel was gently peeled off. Inorder to remove salts and SDS, it was agitated for 15-20 min at room temperature. A piece of nitrocellulose membrane and whatman filter paper was made to the size of the gel which was kept in transfer buffer for 10-15 minutes. A gel holder cassette in a casserole dish was selected which was kept in a position where black side was down. A transfer sandwich (Fig.55) was made inside the gel holder.

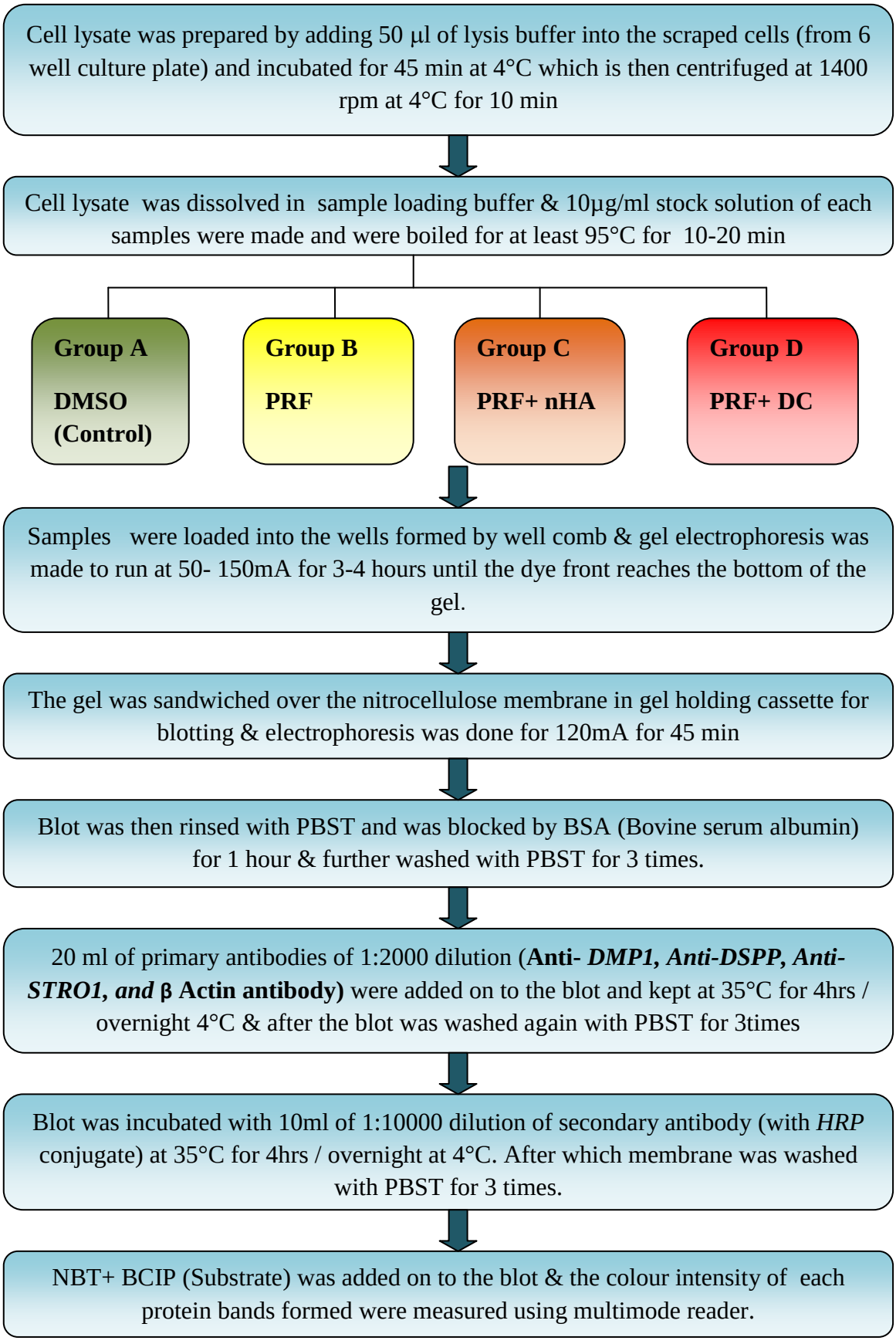



3ml of transfer buffer was added onto the top of each layer and was rolled out with glass tube to prevent air bubbles trapped in. Then the Gel holder cassette was placed in a transfer tank (orient the white and black sides of the cassette with the white and black panels of the electrode) and fill with transfer buffer (~800ml). (Fig.56) Inorder to prevent heat production a styrofoam box containing ice was also placed. The gel electrophoresis was done for 120 mA for 45 min.

**6. Immunodetection**

After electrophoresis the blot was rinsed with wash buffer (PBST) to remove salts and the membrane was blocked by BSA (Bovine serum albumin) for 1 hour. (Fig.57) Further the membrane was washed with PBST for 3 times. Then 20 ml of primary antibodies of 1:2000 dilution (Anti- DMP1, Anti-DSPP, Anti-STRO-1 and β-Actin antibody**)** were added on to the blot and kept at 35°C for 4hrs / overnight 4°C. After which, the blot was washed again with PBST for 3times. Later the blot was incubated at 35°C for 4hrs / overnight at 4°C at with 10ml of 1:10000 dilution of secondary antibody (with *HRP* conjugate ).(Fig.57) After which membrane was washed with PBST for 3 times. The blot was developed with NBT+ BCIP (Substrate). The colour intensity of each protein bands formed (Fig.59) were measured using multimode reader.

**PROCEDURAL FLOW CHART FOR WESTERN BLOT ASSAY**



## MATERIAL

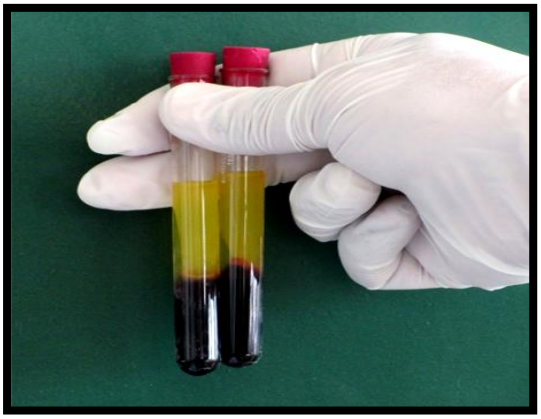


Fig 1. Platelet Rich Fibrin

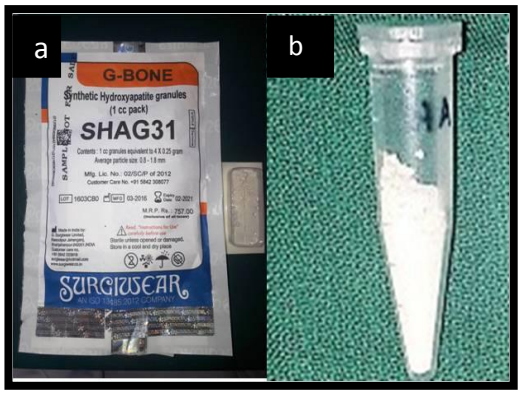



Fig 2. Commercial Nano- hydroxyapatite

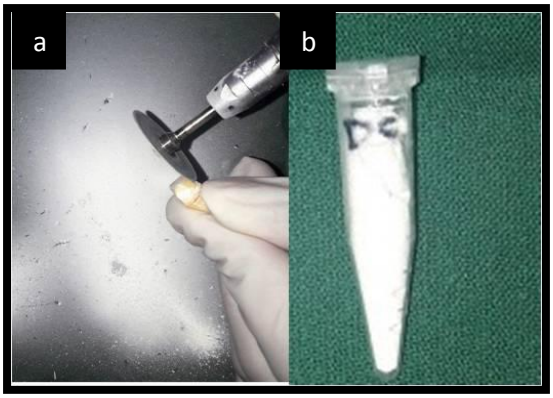



Fig 3. Dentin chips

Fig 4. Dentin disc


Fig 5. DMEM and FBS

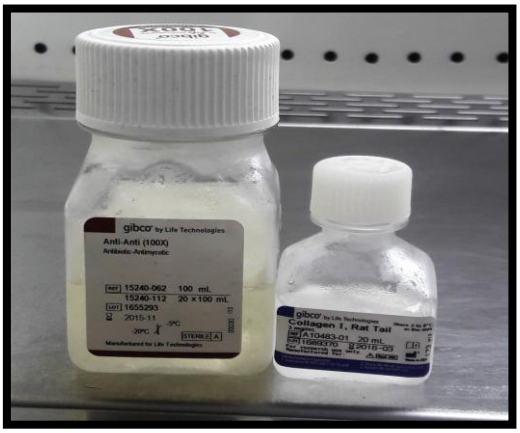



Fig 6. Antibiotic mixture and collagenase type I


Fig 7 Trypsin-EDTA solution

Fig 8 DMSO


Fig 9 MTT assay reagent

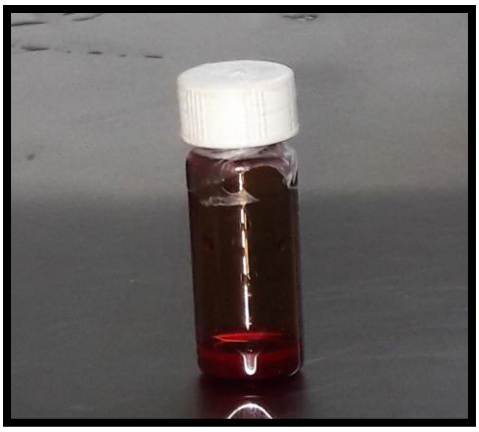


Fig 10 Alizarin red dye


Fig 11. IL-6 & IL-8 antibody


Fig 12. Secondary antibody conjugated with HRP


Fig 13. Nitroblue tetrazolium (NBT) & 5 Bromo-4-chloro-3-indolyl phosphate (BCIP)

**ARMAMENTARIUM**

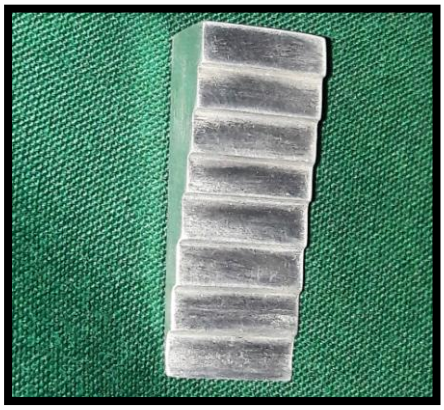


Fig.14 8mm Aluminium step wedge

Fig 15 Metal scale(30cm),Dappen dish, Digital weighing machine, ,Straight handpiece,Watch glass,,SS spatula, Diamond disc,,Mandrel, Glass slab,10ml test tube, 10ml disposable syringe


Fig.16 DIGORA

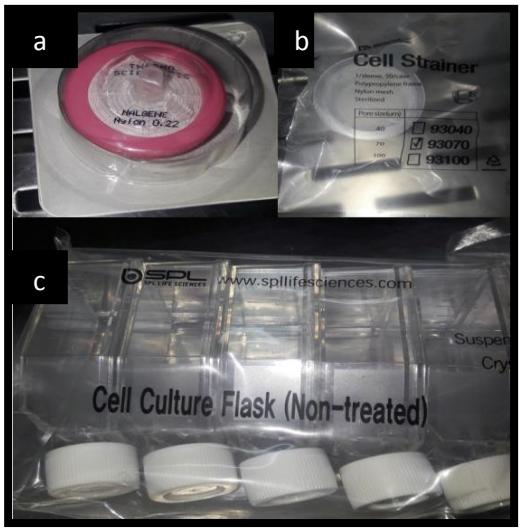



Fig.17 0.22µm Millipore filter,70µm Cell strainer, 25cm$^2$cell culture flask

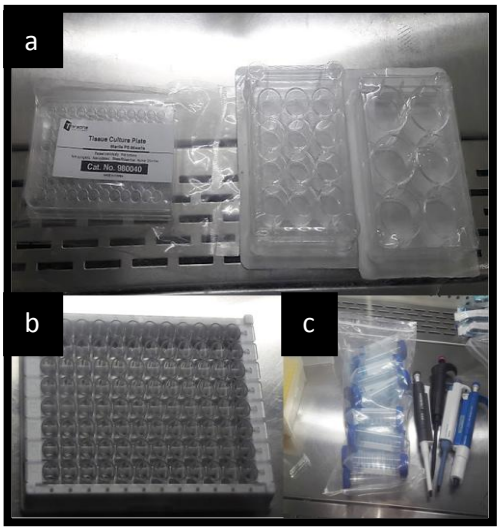



Fig 18 96 well plate, 12 well plate, 6 well plate, ELISA plate, Conical tubes, Micropippetes, Microcentrifuge tubes


Fig.19 Vortexer


Fig.20 Centrifuge

Fig.21 Laminar air flow chamber


Fig. 22 Cell culture $CO_2$ Incubator


Fig.23 Inverted phase contrast microscope

Fig.24 Well comb, spacer, glass plates

Fig 25 Gel cassette holder


Fig 26 Vertical electrophoresis unit

Fig 27 Multimode plate reader unit

**Measuring aluminium equivalent of radiopacity**


Fig.28 Weighing PRF & dentin chip, nHA using digital weighing machine

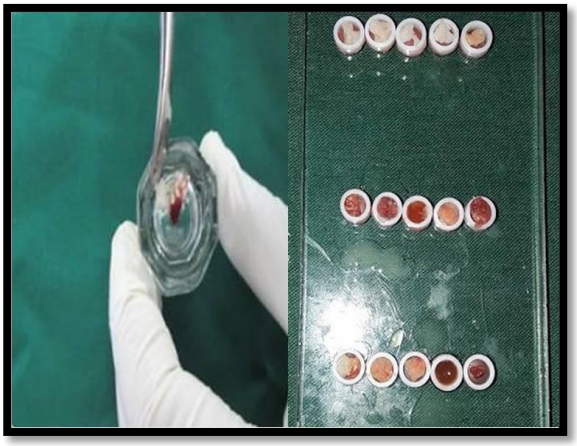


Fig.29 Trituration of PRF+ nHA/DC, compacted into Teflon rings of 2mm height

Fig. 30 Samples radiographed along with 8mm aluminium step wedge


Fig. 31a. Radiopacity measured using DIGORA for windows 2.9.113.490

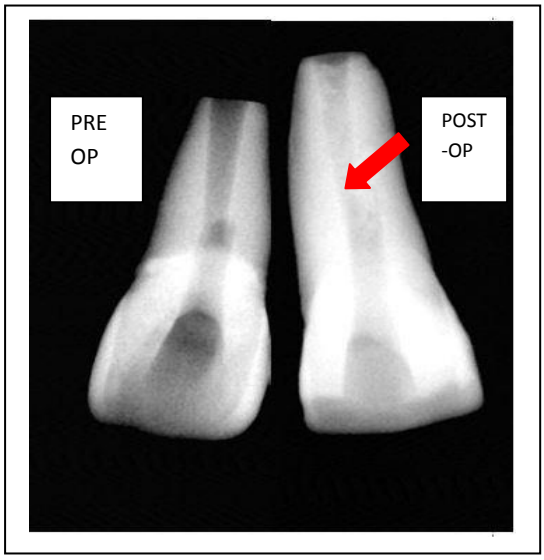



Fig. 31.b Pre-OP radiograph of 11(Left-side) with an open apex . Post-OP (Right- side) radiograph shows increased amount of radiopacity obtained within root canal when PRF+50wt% nHA used as apexification material

**Enzymatic isolation human dental pulp stem cells**

Fig 32 Extirpated Pulp tissue

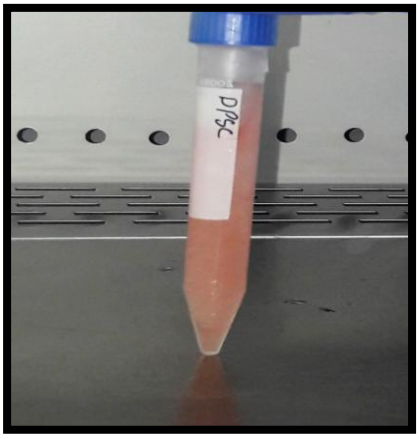


Fig 33 Pulp tissue transported in DMEM-FBS medium

Fig 34 Pulp tissue minced with scalpel

Fig 35. Centrifugation of minced pulp tissue at 1200 rpm, 10 min


Fig 36 Pelleted pulp tissue

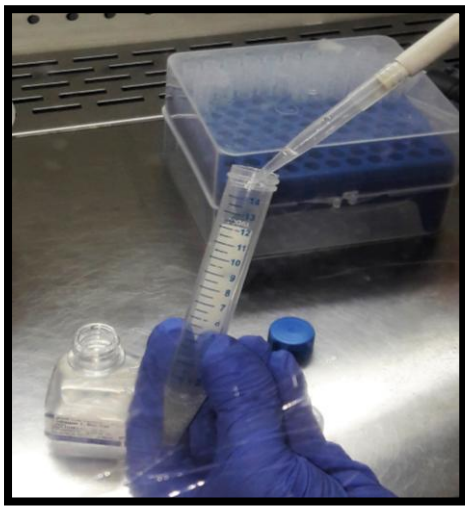


Fig. 37 Pelleted tissue digested with 3mg/ml Collagenase type I for 60 min , 37°C& centrifuged at 1200 rpm for 10 min

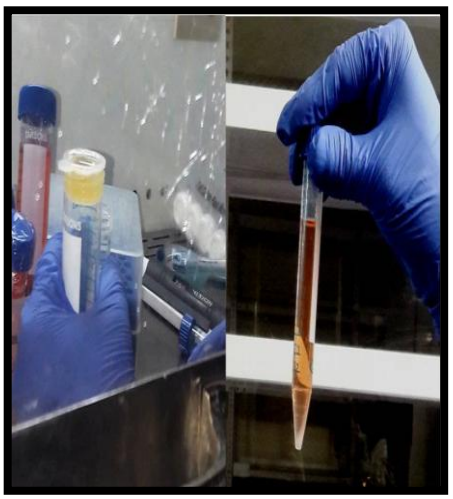


Fig. 38 Solution filtered through cell strainer & Cell pellet resuspended in FBS (10%) containing medium

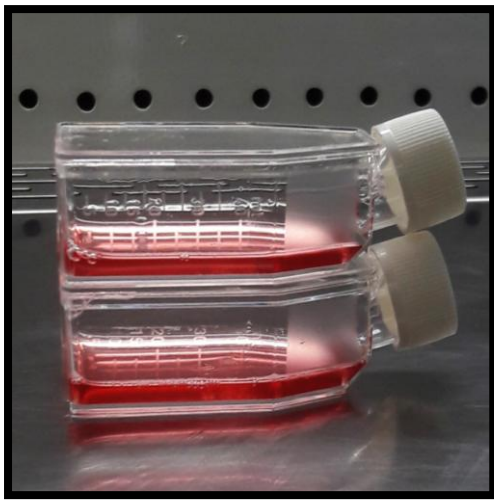


Fig.39 Single cell suspension were then resuspended DMEM+FBS(10%) & transferred to T25 flasks

Fig 40 Suspended cells in T25 flask cultured at 5% $CO_2$ at 37°C

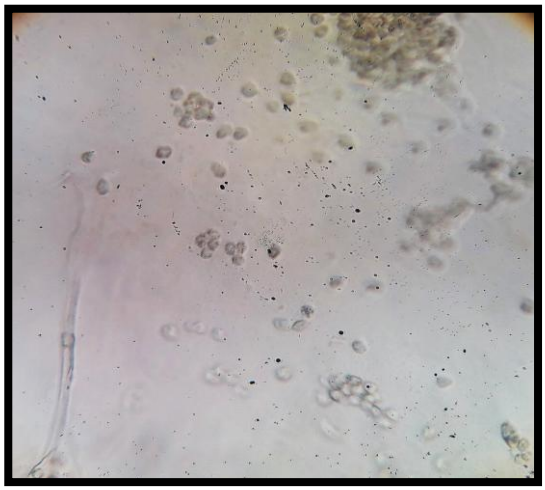


Fig 41 Cells seen immediately after culture under phase contrast microscope -10X

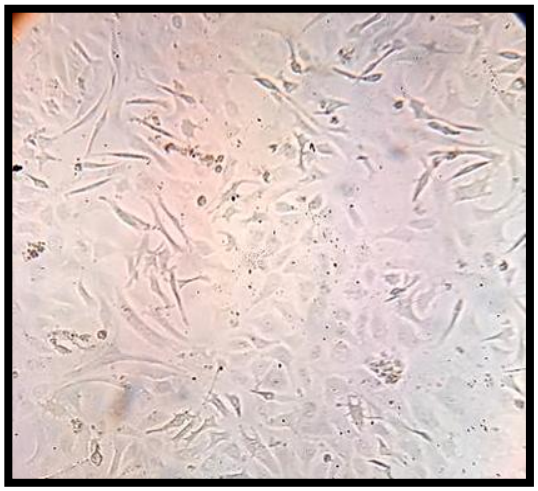


Fig.42 4 days after culture under phase contrast microscope -10X

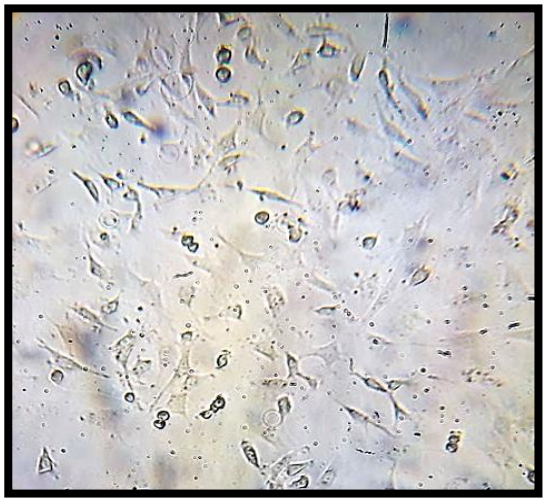


Fig 43 10 days after culture under phase contrast microscope- 40X

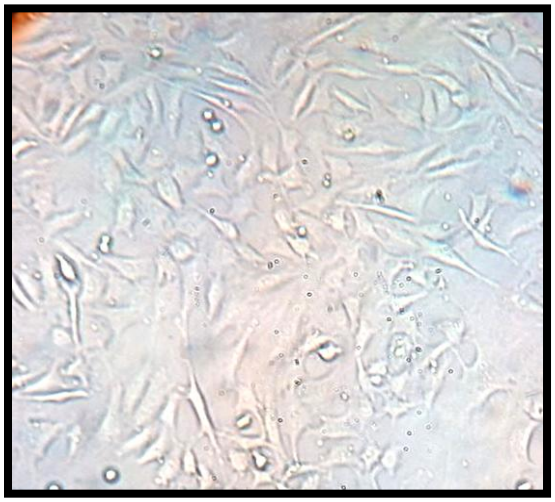


Fig .44 2weeks culture under phase contrast microscope-40X

**MTT ASSAY**

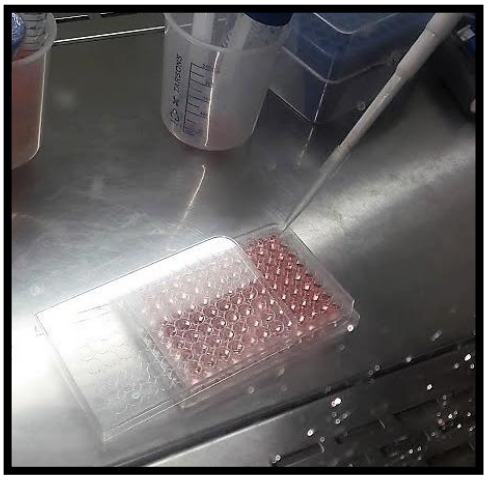


Fig 45 Seeding of cells into 96 well plate

Fig. 46 Addition of MTT dye

**ELISA**

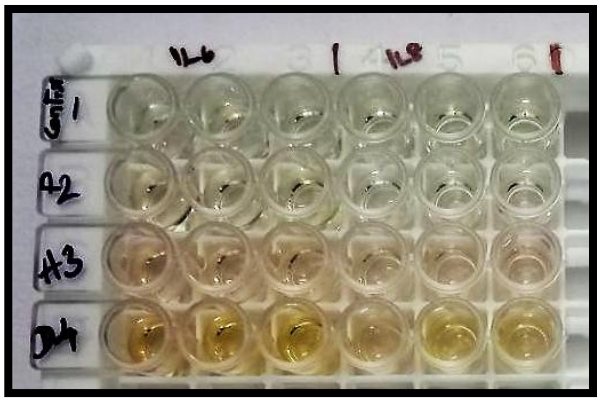


Fig.47 ELISA plate with samples after addition of substrate

**MINERALIZATION ASSAY**

Fig.48 Adding Alizarin red dye into 12 well plate

**WESTERN BLOT**

Fig.49 Seeding of cells into 6 well culture plate

Fig. 50 Detached cells collected by PBS wash & centrifuged at 5000 rpm for 5 min at 4 ºC

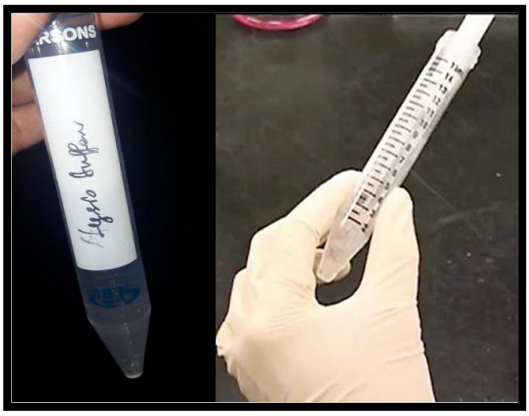



Fig. 51 Cells resuspended in lysis buffer & incubated for 45 min at 4 ºC, later centrifuged

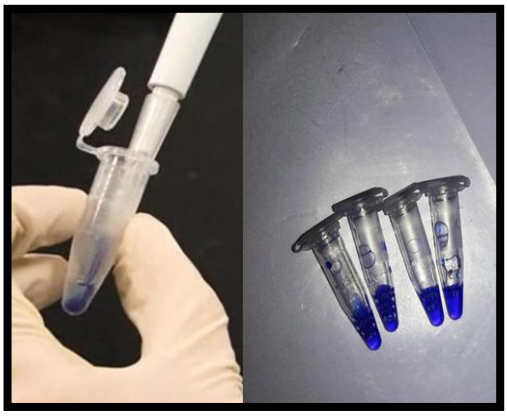


Fig.52 Sample loading buffer added to samples and boiled at 95°C for 10 min


Fig .53 Samples added into wells.Gel electrophoresis done at 120mA.

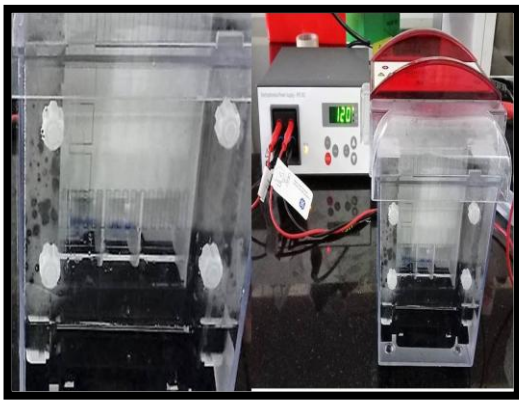


Fig.54 Gel electrophoresis at the end of 3hrs

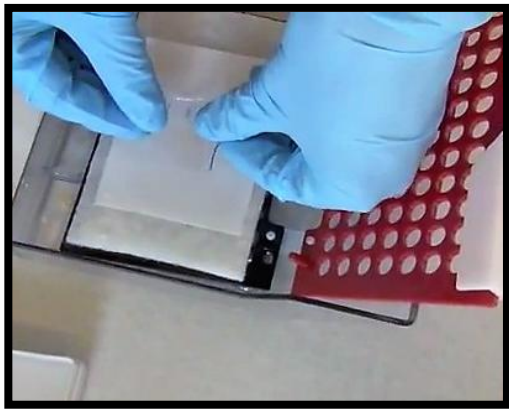


Fig.55 Gel sandwiched over the nitrocellulose membrane in gel holding cassette for blotting

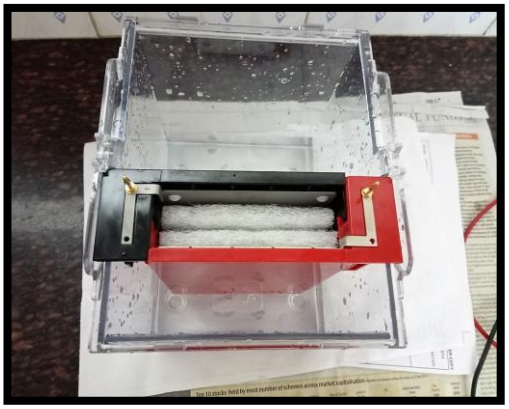


Fig. 56 Sandwich (gel& nitrocellulose membrane) transferred to transfer tank for electrophoresis

Fig. 57 Membrane blocked with BSA

Fig.58 Primary & Secondary antibody(with HRP) added and incubated

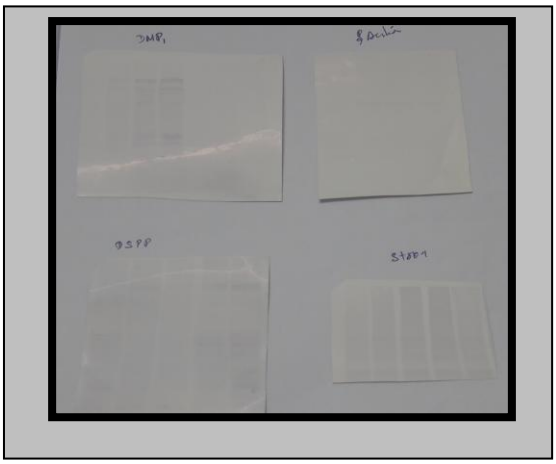


Fig .59 Coloured protein bands formed after adding NBT+ BCIP (Substrate)

## STATISTICAL ANALYSIS

The values were recorded for each groups. The values were analysed statistically by One Way analysis of Variance (for intragroup analysis) followed by multiple comparison Tukey HSD test (for intergroup analysis). All statistical analysis were done using SPSS VERSION 23. Statistical significance was determined at $P < 0.05$.

### I. ALUMINIUM EQUIVALENT OF RADIOPACITY ANALYSIS

**Table 1(a-d): Radiopacity density values obtained by each group using digora software converted to aluminium equivalents**

**a) Group 1(Dentin disc)**

| Sample | | | | | | mean | mmAl |
|---|---|---|---|---|---|---|---|
| Sample 1 | 50.99 | 49.32 | 56.22 | 50.23 | 49.32 | 51.21 | 2.62 |
| Sample 2 | 49.02 | 54.44 | 53.41 | 52.34 | 52.4 | 52.32 | 2.66 |
| Sample 3 | 54.12 | 49.04 | 48.32 | 53.3 | 55.38 | 52.03 | 2.65 |

**b) Group 2 (PRF)**

| Samples | | | | | | mean | mmAl |
|---|---|---|---|---|---|---|---|
| Sample 1 | 7.01 | 15 | 5.01 | 10.34 | 6.7 | 7.6 | 0.26 |
| Sample 2 | 8.8 | 9.56 | 8.34 | 8.43 | 7.56 | 8.5 | 0.29 |
| Sample 3 | 6.04 | 5.41 | 9.8 | 7.4 | 6.32 | 6.99 | 0.241 |
| Sample 4 | 8.57 | 7.88 | 12.01 | 5.44 | 7.5 | 8.28 | 0.285 |
| Sample 5 | 8.24 | 5.04 | 6.66 | 11.42 | 5.01 | 7.27 | 0.25 |

**c) Group 3 (PRF+50wt%nHA)**

| Samples | | | | | | mean | mmAl |
|---|---|---|---|---|---|---|---|
| Sample 1 | 49.8 | 38.4 | 42.12 | 41.96 | 39.92 | 42.44 | 1.52 |
| Sample 2 | 43.44 | 42.54 | 37.32 | 39.06 | 38.33 | 40.13 | 1.39 |
| Sample 3 | 45.88 | 42.66 | 43.38 | 45.42 | 40.78 | 43.62 | 1.59 |
| Sample 4 | 46.23 | 41.44 | 39.92 | 47.24 | 45.06 | 43.98 | 1.61 |
| Sample 5 | 42.53 | 45.32 | 40.12 | 40.92 | 39.21 | 41.62 | 1.47 |

**d) Group 4 (PRF+50wt%DC)**

| Samples | | | | | | mean | mmAl |
|---|---|---|---|---|---|---|---|
| Sample 1 | 25.49 | 32.55 | 28.63 | 34.27 | 36.47 | 31.42 | 0.95 |
| Sample 2 | 28.71 | 34.06 | 34.9 | 28.54 | 30.49 | 31.34 | 0.94 |
| Sample 3 | 34.87 | 32.22 | 34.86 | 30.92 | 38.02 | 34.98 | 0.97 |
| Sample 4 | 27.93 | 30.87 | 32.18 | 33.48 | 39.62 | 32.81 | 0.99 |
| Sample 5 | 38.78 | 31.11 | 30.54 | 31.74 | 31.36 | 32.7 | 0.99 |

**Table 2a: Descriptive analysis of Aluminium equivalent obtained for each group**

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| Group 1 | 3 | 2.6433 | .02082 | .01202 | 2.5916 | 2.6950 | 2.62 | 2.66 |
| Group 2 | 5 | .2652 | .02151 | .00962 | .2385 | .2919 | .24 | .29 |
| Group 3 | 5 | 1.5160 | .08989 | .04020 | 1.4044 | 1.6276 | 1.39 | 1.61 |
| Group 4 | 5 | .9680 | .02280 | .01020 | .9397 | .9963 | .94 | .99 |
| Total | 18 | 1.2042 | .81976 | .19322 | .7966 | 1.6119 | .24 | 2.66 |

**Table 2b: ANOVA analysis showing F value for Aluminium equivalent between groups and within groups**.

| Radiopacity | | | | | |
|---|---|---|---|---|---|
| | Sum of Squares | df | Mean Square | F | Sig. |
| Between Groups | 23017.687 | 3 | 7672.562 | 867.031 | .000 |
| Within Groups | 761.034 | 86 | 8.849 | | |
| Total | 23778.721 | 89 | | | |

* The mean difference is significant at the 0.05 level

**Table 2c: Tukey Post Hoc test for intergroup comparative analysis of aluminium equivalent of radiopacity**

| (I) Groups3 | (J) Groups3 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound |
| Group 1 | Group 2 | 2.37813* | .03760 | .000 | 2.2688 | 2.4874 |
| | Group 3 | 1.12733* | .03760 | .000 | 1.0180 | 1.2366 |
| | Group 4 | 1.67533* | .03760 | .000 | 1.5660 | 1.7846 |
| Group 2 | Group 1 | -2.37813* | .03760 | .000 | -2.4874 | -2.2688 |
| | Group 3 | -1.25080* | .03257 | .000 | -1.3455 | -1.1561 |
| | Group 4 | -.70280* | .03257 | .000 | -.7975 | -.6081 |
| Group 3 | Group 1 | -1.12733* | .03760 | .000 | -1.2366 | -1.0180 |
| | Group 2 | 1.25080* | .03257 | .000 | 1.1561 | 1.3455 |
| | Group 4 | .54800* | .03257 | .000 | .4533 | .6427 |
| Group 4 | Group 1 | -1.67533* | .03760 | .000 | -1.7846 | -1.5660 |
| | Group 2 | .70280* | .03257 | .000 | .6081 | .7975 |
| | Group 3 | -.54800* | .03257 | .000 | -.6427 | -.4533 |

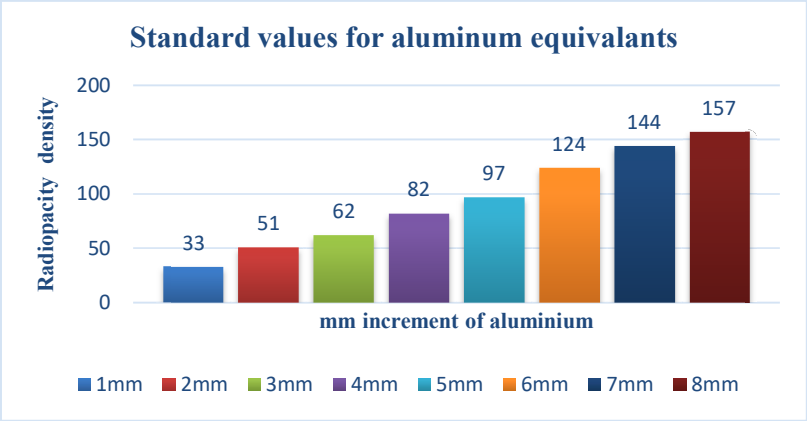
*The mean difference is significant at the 0.05 level

**Interpretation**

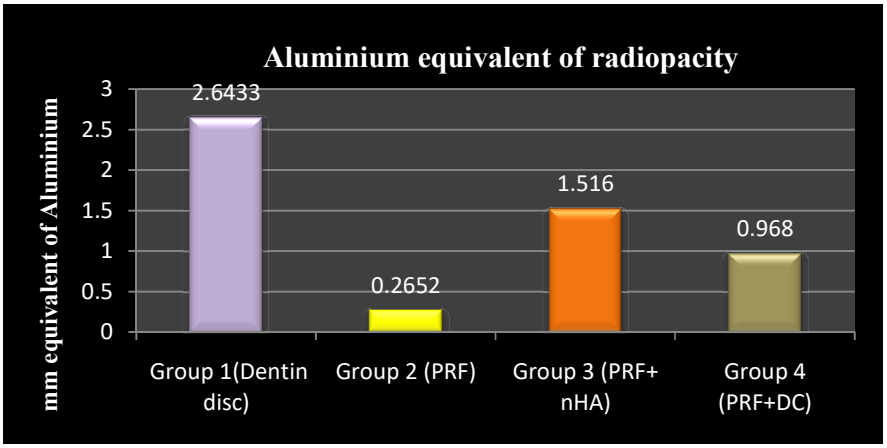
One way ANOVA analysis showed high significant statistical difference in aluminium equivalent of radiopacity between groups and within the groups. (P<0.001)

On multiple comparison of groups done by Tukey post hoc test showed a high stastical significant differences in aluminium of equivalent of radiopacity between 4 groups (P<0.001)

**Graphs:**




**Graph. 1 represents mean radiopacity density values for 1mm increment for aluminium step wedges (plotted for 8mm aluminium step wedge) with x axis representing thickness of aluminium wedges with increase of 1mm increment and y axis representing corresponding radiopacity density score.**




**Graph 2 represents mean aluminium equivalent of radiopacity of each group, where Group 1 (control- Dentin Disc) is showing highest mm equivalent of aluminium and least was Group 2 (PRF). The value of Group 3 (PRF+ 50wt% nHA) is almost half the density of Group 1(control- Dentin Disc)**

## II. CELL CULTURE BASED ASSAY ANALYSIS

### 1. MTT ASSAY

**Table 3 (a&b): shows optical densities of each group in 96 well plate, obtained using multimode plate reader**

**a) Group A- DMSO (control)**

| Conc | OD1 | OD2 | Mean | %cytotoxicity |
|---|---|---|---|---|
| 1µg/ml | 0.831 | 0.823 | 0.827 | 1.371497 |

**b) Group B (PRF) , Group C (PRF+ nHA), Group D (PRF+ DC)**

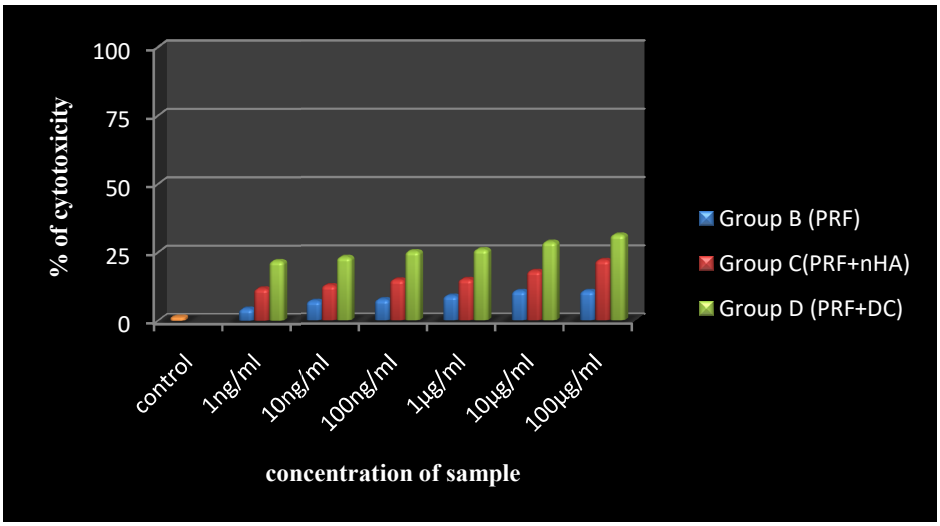
| GROUP B | | | | | GROUP C | | | | GROUP D | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CONC | OD 1 | OD 2 | Mean | % cyt | OD1 | OD2 | Mean | % cyt | OD1 | OD2 | Mean | % cyt |
| 1ng/ Ml | 0.805 | 0.798 | 0.801 | 4.412 | 0.742 | 0.737 | 0.739 | 11.80 | 0.664 | 0.641 | 0.647 | 21.86 |
| 10ng/ ml | 0.781 | 0.776 | 0.778 | 7.155 | 0.736 | 0.725 | 0.730 | 12.88 | 0.652 | 0.636 | 0.644 | 23.19 |
| 100ng/ ml | 0.778 | 0.769 | 0.773 | 7.751 | 0.721 | 0.705 | 0.713 | 14.96 | 0.631 | 0.621 | 0.626 | 25.34 |
| 1µg/ Ml | 0.768 | 0.757 | 0.762 | 9.063 | 0.712 | 0.711 | 0.711 | 15.14 | 0.621 | 0.619 | 0.62 | 26.05 |
| 10µg/ ml | 0.754 | 0.742 | 0.748 | 10.79 | 0.754 | 0.742 | 0.748 | 18.12 | 0.602 | 0.593 | 0.597 | 28.74 |
| 100µg/ ml | 0.752 | 0.741 | 0.746 | 10.79 | 0.752 | 0.741 | 0.746 | 22.12 | 0.581 | 0.569 | 0.575 | 31.42 |

**Table 4: An Intragroup comparison by One way ANOVA shows mean, standard deviation obtained at each concentrations for different groups**

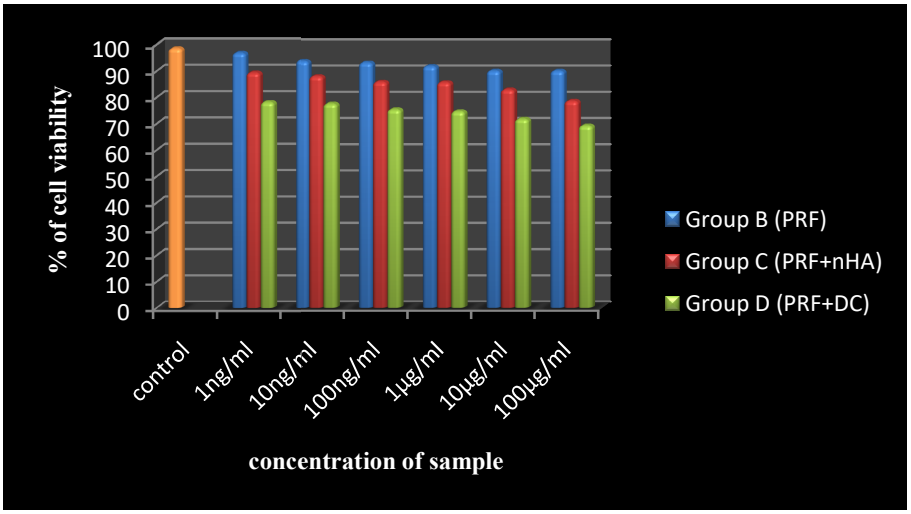
| | A | | B | | C | | D | | F value | P value |
|---|---|---|---|---|---|---|---|---|---|---|
| Concentration | Mean | SD | Mean | SD | Mean | SD | Mean | SD | | |
| 1ng/ml | 0 | 0 | .802 | .005 | .740 | .004 | .653 | .016 | 144.669 | <0.001** |
| 10ng/ml | 0 | 0 | .779 | .004 | .731 | .008 | .644 | .011 | 138.689 | 0.001* |
| 100ng/ml | 0 | 0 | .774 | .006 | .713 | .011 | .626 | .007 | 150.963 | 0.001* |
| 1µg/ml | .827 | .006 | .763 | .008 | .712 | .001 | .620 | .001 | 496.500 | <0.001** |
| 10µg/ml | 0 | 0 | .748 | .008 | .687 | .008 | .598 | .006 | 198.575 | 0.001* |
| 100µg/ml | 0 | 0 | .747 | .008 | .653 | .007 | .575 | .008 | 242.403 | <0.001** |

**-Highly significant (p<0.001), *-significant (p<0.05)

**Graphs:**




**Graph 3 represents comparison of percentage of cytotoxicity (y axis) within each concentration of samples (x axis) between Group B, C, D compared to control (Group A). % of cytotoxicity was least for control (Group A) followed by Group B (PRF) > Group C (PRF+nHA ) > Group D(PRF+DC).**




**Graph 4 represents comparison of percentage of cell viability within each concentration of sample between Group B, C, D compared to control (Group A). In Group B (PRF) 90% of cells were viable. Group C (PRF+ nHA) and Group D (PRF+DC) has >73% viable cells. (Fig.60-63)**

**2. ELISA**

**Table 5: Optical densities obtained in each group in 24 well ELISA plate read by multimode plate reader which represents levels of expression of IL-6 & IL-8**

| | IL-6 | | | IL-8 | | |
|---|---|---|---|---|---|---|
| | OD1 | OD2 | MEAN | OD1 | OD2 | MEAN |
| Group A (Control) | 0.106 | 0.116 | 0.1635 | 0.115 | 0.105 | 0.110 |
| Group B(PRF) | 0.167 | 0.160 | 0.111 | 0.175 | 0.177 | 0.175 |
| Group C (PRF+nHA) | 0.176 | 0.188 | 0.1818 | 0.193 | 0.183 | 0.18805 |
| Group D (PRF+DC) | 0.267 | 0.266 | 0.2665 | 0.346 | 0.283 | 0.3145 |

**Table 6a: Descriptive analysis for optical densities exhibited by each group for IL6 and IL-8**

| | | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound | | |
| IL6 | Control | 2 | .1110 | .00707 | .00500 | .0475 | .1745 | .11 | .12 |
| | PRF | 2 | .1635 | .00495 | .00350 | .1190 | .2080 | .16 | .17 |
| | PRF + HA | 2 | .1820 | .00849 | .00600 | .1058 | .2582 | .18 | .19 |
| | PRF + DC | 2 | .2665 | .00071 | .00050 | .2601 | .2729 | .27 | .27 |
| | Total | 8 | .1808 | .05998 | .02121 | .1306 | .2309 | .11 | .27 |
| IL8 | Control | 2 | .1100 | .00707 | .00500 | .0465 | .1735 | .10 | .12 |
| | PRF | 2 | .1760 | .00141 | .00100 | .1633 | .1887 | .18 | .18 |
| | PRF + HA | 2 | .1880 | .00707 | .00500 | .1245 | .2515 | .18 | .19 |
| | PRF + DC | 2 | .3145 | .04455 | .03150 | -.0857 | .7147 | .28 | .35 |
| | Total | 8 | .1971 | .08096 | .02862 | .1294 | .2648 | .10 | .35 |

**Table 6b: Tukey Post Hoc test for intergroup comparative analysis of optical densities for variable IL-6 and IL-8**

| Dependent Variable | (I) Groups1 | (J) Groups1 | Mean Difference (I-J) | Std. Error | Sig. | 95% Confidence Interval | |
|---|---|---|---|---|---|---|---|
| | | | | | | Lower Bound | Upper Bound |
| IL6 | Control | PRF | -.05250* | .00606 | .013 | -.0772 | -.0278 |
| | | PRF + HA | -.07100* | .00606 | .001 | -.0957 | -.0463 |
| | | PRF + DC | -.15550* | .00606 | .000 | -.1802 | -.1308 |
| | PRF | Control | .05250* | .00606 | .003 | .0278 | .0772 |
| | | PRF + HA | -.01850 | .00606 | .119 | -.0432 | .0062 |
| | | PRF + DC | -.10300* | .00606 | .000 | -.1277 | -.0783 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | PRF + HA | Control | .07100* | .00606 | .001 | .0463 | .0957 |
| | | PRF | .01850 | .00606 | **.119** | -.0062 | .0432 |
| | | PRF + DC | -.08450* | .00606 | .001 | -.1092 | -.0598 |
| | PRF + DC | Control | .15550* | .00606 | .000 | .1308 | .1802 |
| | | PRF | .10300* | .00606 | .000 | .0783 | .1277 |
| | | PRF + HA | .08450* | .00606 | .001 | .0598 | .1092 |
| IL8 | Control | PRF | -.06600 | .02284 | **.138** | -.1590 | .0270 |
| | | PRF + HA | -.07800 | .02284 | **.086** | -.1710 | .0150 |
| | | PRF + DC | -.20450* | .02284 | .003 | -.2975 | -.1115 |
| | PRF | Control | .06600 | .02284 | **.138** | -.0270 | .1590 |
| | | PRF + HA | -.01200 | .02284 | **.948** | -.1050 | .0810 |
| | | PRF + DC | -.13850* | .02284 | .013 | -.2315 | -.0455 |
| | PRF + HA | Control | .07800 | .02284 | **.086** | -.0150 | .1710 |
| | | PRF | .01200 | .02284 | **.948** | -.0810 | .1050 |
| | | PRF + DC | -.12650* | .02284 | .018 | -.2195 | -.0335 |
| | PRF + DC | Control | .20450* | .02284 | .003 | .1115 | .2975 |
| | | PRF | .13850* | .02284 | .013 | .0455 | .2315 |
| | | PRF + HA | .12650* | .02284 | .018 | .0335 | .2195 |

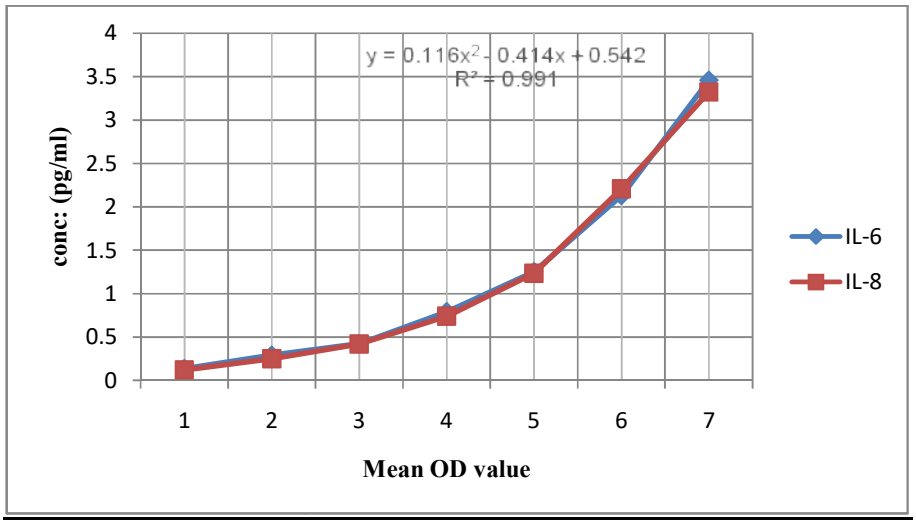
*. The mean difference is significant at the 0.05 level.

**Interpretation**

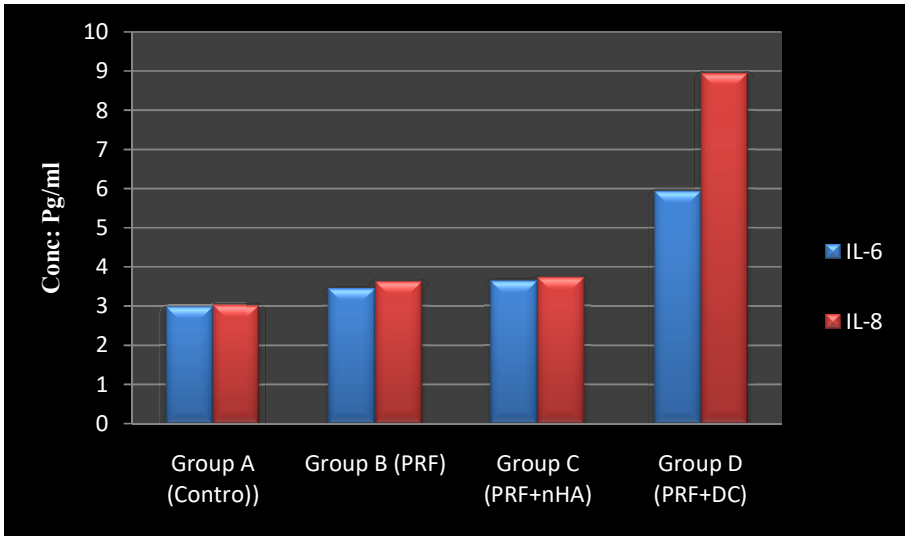
For IL-6 scores, showed there was significant difference between Group A (Control) with Group B (PRF), Group C (PRF+nHA), Group D (PRF+DC) ($p<0.05$). There was no significant difference between Group B (PRF) & Group C (PRF+nHA)

For IL-8 scores, significant difference was there between Group D (PRF+DC) with Group A (control), Group B (PRF) & Group C (PRF+ nHA) with $p<0.05$.

**Graphs:**




**Graphs 5 represents standard values for IL-6 and IL-8, where mean OD values (x axis ) plotted against different concentrations (y axis) of IL-6 & IL-8 (pg/ml)**




**Graph 6 represents the mean OD value for IL-6 and IL-8 given by different groups where mean OD values (x axis) obtained for each group (at conc: µg/ml) plotted against standard concentration of IL-6 & lL-8 ( pg/ml).**

### 3. MINERALIZATION ASSAY (Fig.64-67)

In phase contrast microscopic view of (at 40x magnification) alizarin red staining of samples showed a orange red stained nodules in Group B (PRF), Group C (PRF+ nHA) and Group D (PRF+DC). There was appreciable number of mineralization nodules in Group C (PRF+ nHA), compared to Group B (PRF) and Group D (PRF+ DC).

### 4. WESTERN BLOT ASSAY

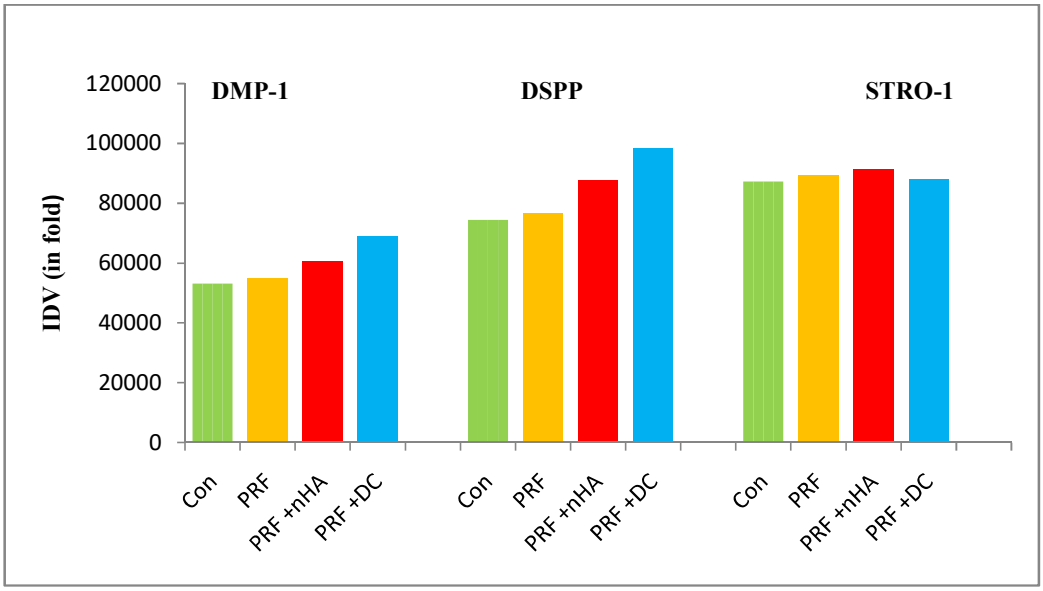
**Table 7: Intergrated density values obtained for expression each protein band (DMP1, DSPP , STRO-1 and β-Actin) using image J density software. (Fig. 68)**

| | DMP1 | DSPP | STRO-1 | β-Actin |
|---|---|---|---|---|
| Group A | 53080.69 | 74391.11 | 87261.71 | 50653.78 |
| Group B | 55043.45 | 76637.24 | 89566.76 | 53288.91 |
| Group C | 60721.49 | 87870.66 | 91582.32 | 52184.07 |
| Group D | 68921.19 | 98343.45 | 88055.26 | 53657.23 |

**Interpretation**

Increased expression of protein bands of STRO-1 in Group B (PRF), Group C (PRF+nHA), Group D (PRF+DC) compared to Group A (Control)

Increased expression of DMP-1 and DSSP in Group C (PRF+ nHA) and Group D (PRF+DC) compared to Group A (Control) and Group B (PRF).




**Graph 7 represents Integrated density values (y axis) obtained for expression each protein band ( DMP1, DSPP , STRO-1) for 4 groups .**

**MTT ASSAY**

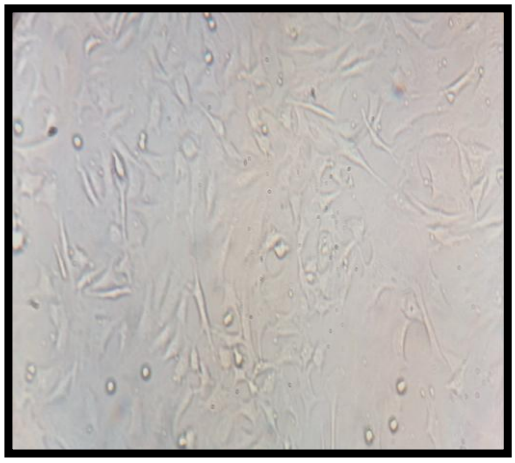


Fig .60 Group A- Control

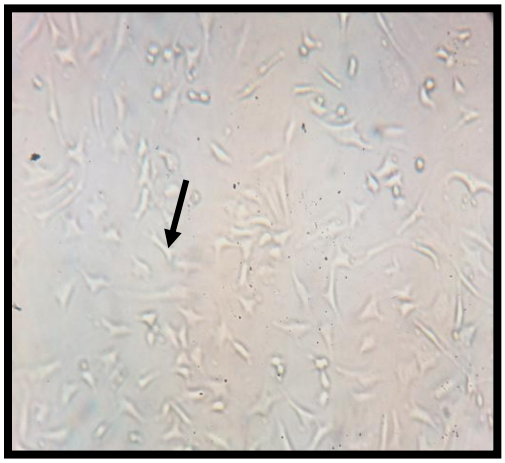


Fig.61 Group B- PRF

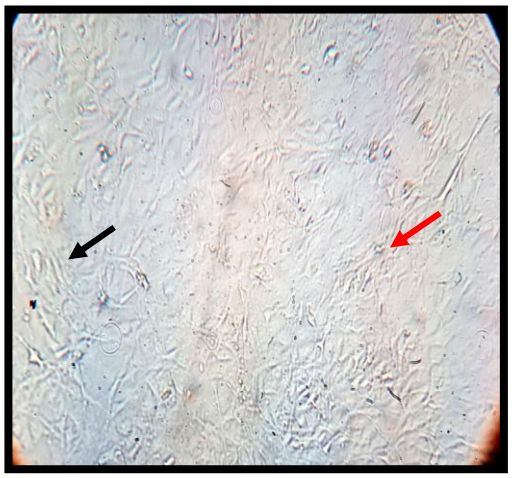


Fig 62 Group C- PRF + nHA

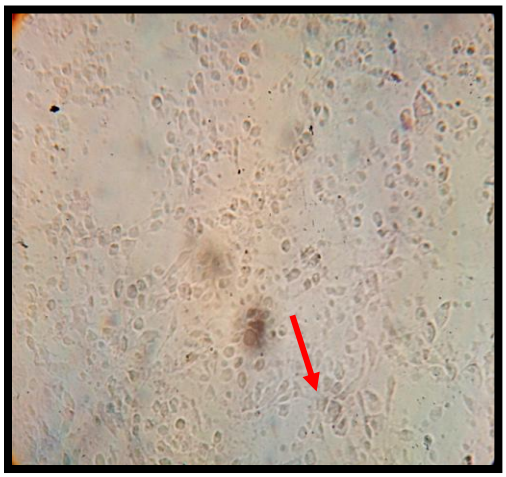


Fig 63 Group D- PRF+DC

NB: Black arrow indicates the cells with normal fibroblastic/spindle shaped morphology & red arrow indicates the cells with lost morphology, indicates the cytotoxicity

## MINERALIZATION ASSAY

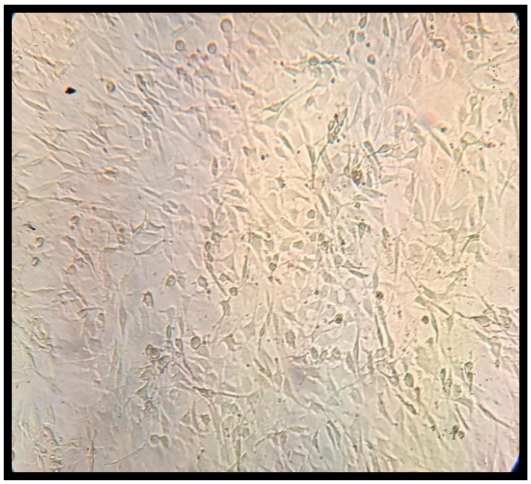


Fig .64 Group A- Control

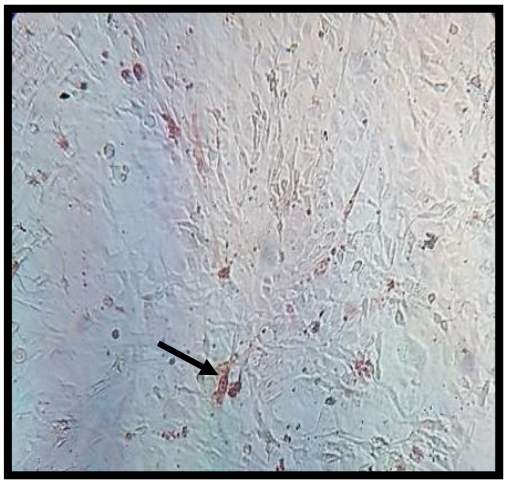


Fig .65 Group B- PRF

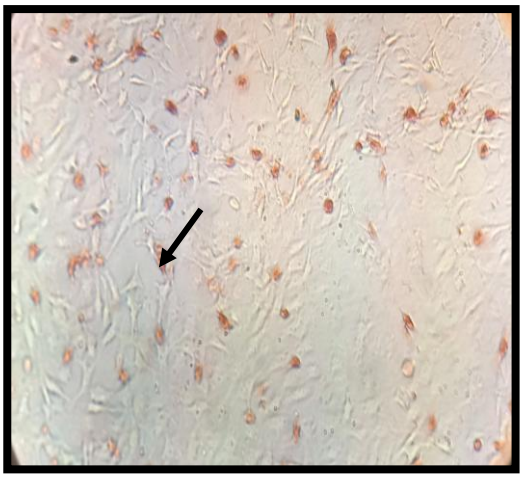


Fig .66 Group C- PRF+ nHA

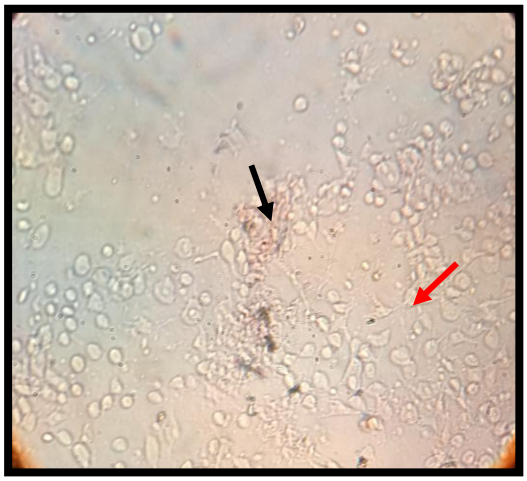


Fig .67 Group D- PRF+DC

N.B Black arrow indicates the orange red stained mineralization nodule, Red arrow indicates the inflamed cells with loss of normal fibroblastic appearance.

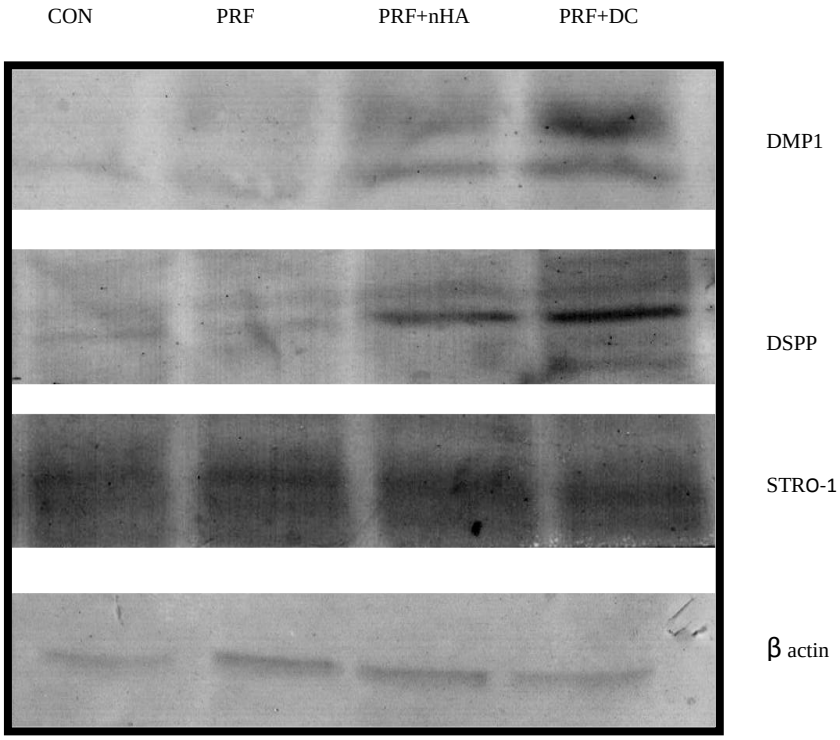



Fig. 68 Protein bands

## DISCUSSION

Regenerative endodontics is concerned with the development of biologically based treatment modalities that are used to replace diseased portions of the dental pulp or to allow complete formation of a dental pulp–like tissue that will act as the original dental pulp.[27] This concept of tissue regeneration made a revolution in field of endodontics & one of the key components of concept is the utilization of scaffold. Advancement in this field has helped to create a way to use the patient's own tissue to repair and regenerate various bony and soft-tissue lesions. In consequence, a rapidly growing number of studies have explored the efficacy of supplementing stem cell based tissue engineering approaches with natural growth factor cocktails such as platelet concentrates. This has paved the way for improvements in stem cell function including cell growth, viability, proliferation, differentiation and overall regenerative potential.

Platelet concentrates have gained immense popularity as bioactive surgical additives in the field of regenerative dentistry. In 2001, Choukroun & co-workers introduced the protocol for PRF (second generation platelet concentrate), in which slow and natural polymerization of fibrin resulted in homogenous 3-dimensional organization.[20] It is made up of fine, flexible, mature & dense polymerized fibrin strands.[20] This leads to the intrinsic incorporation of platelet cytokines and glycan chains in the fibrin meshes.[6] Thus the use of PRF addresses two parts of the triad for tissue regeneration-growth factors and a scaffold.

Three major modifications (**A-PRF** by Ghanaati 2014 , **A-PRF+** by Fujioka–Kobayashi, 2016 & **I-PRF** by Mourao, 2015) of the PRF technique have been suggested in the protocol over the last few years, but on literature search there was no

long term evidences to prove their success.[83] So we have chosen L-PRF (Leukocyte rich PRF concentrate) in our study, which was formed when human blood centrifuged at 3000 rpm for 10min. The variations are in protocol related to the revolutions or time or the material of the tube. [83]

According to Simonpieri et al (2009), the use of this Leukocyte rich PRF concentrate (L-PRF) offers the following 4 advantages.[86] First, the fibrin clot plays an important mechanical role, with the PRF membrane maintaining and protecting the grafted biomaterials and PRF fragments serving as biological connectors between bone particles. Second, the integration of this fibrin network into the regenerative site facilitates cellular migration, particularly for endothelial cells necessary for the neo-angiogenesis, vascularization and survival of the graft. Third, the platelet cytokines (PDGF, TGF-β, IGF-1) will be gradually released when the fibrin matrix get resorb, thus creating a perpetual process of healing. Lastly, the presence of leukocytes and cytokines in the fibrin network can play a significant role in the self-regulation of inflammatory and infectious phenomena within the grafted material.

Development of PRF became a novel acumen in field of regenerative endodontics where it is used in revascularisation procedures, regenerative pulptomies and to fill the bony defects in surgical procedures. Ray et al (2016)[77] illustrated a revascularization protocol through a case where platelet-rich fibrin (PRF) was utilized as an autologous scaffold for traumatized, necrotic, immature teeth-21 with incomplete root development. On 36 month follow-up, 21 tested positive to EPT testing and radiograph showed an increase in root length with an intact lamina dura.

One of the clinical successs of apexification / regenerative procedure is the precise placement of material inside the root canal. When reviewing a immediate

postoperative radiograph, it is important to ensure that the material is: 1) within the root canal; 2) well packed without defects; 3) discernible from dentin; 4) discernible from other filling material used and 5) distinguishable from superimposed bone trabeculae. For placement of scaffold material inside the root canal, one has to measure and confirm the working length with master cone radiograph. But PRF lacks significant radiopacity to be dicernible in the radiograph, which can compromise its precise placement to the working length of root canal.

There are various commercially available radiopacifiers which are used to provide radiopacity to dental materials. Duarte et al (2009)[37] evaluated the radiopacifying capacity of different radiopacifiers bismuth oxide, zinc oxide, lead oxide, bismuth subnitrate, bismuth carbonate, barium sulfate, iodoform, calcium tungstate, and zirconium oxide associated with Portland cement. A ratio of 20% radiopacifier and 80% white Portland cement by weight was used for analysis. All tested substances presented higher radiopacity than that of dentin and satisfied the ISO criteria of radiopacity for root end filling material (at least 3mm equivalent of Al)[81] & criteria for radiopacity for root canal sealer by ANSI/ADA specification no. 571 (atleast 2mm equivalent of aluminium)[93]. But these radiopacifiers are being heavy metals it remained as a question to use them along with a bio-scaffold (PRF), which might have a possible interference with its biocompatibility and physical properties. In addition, these radiopacifiers reported to have slow resorption rates which lead to residual radiopacity.[69] Residual radiopacity conceal the radiographic interpretation of healing.

When we did an alternate search, found that calcium phosphate material which are bioactive in nature can serve the purpose as a radiopacifier. Hence we have chosen 2

bioactive materials (Nano- hydroxyapatite and Dentin chips) which are easily available.

**Nanohydroxyapatite (*G-Bone, nHA*)** is synthetic hydroxyapatite granules and blocks which are made of Multiphasic Calcium Hydroxyapatite in low crystalline form.[70] It is derived from bovine bone which is sintered at +500 Celsius and this high temperature leaves only pure inorganic structure and thus removes the risk of transmission of any disease.[70] Hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$] has its similarity to the composition of the bone structure (about 60% is calcium phosphate). Synthetic apatite demonstrates good biological properties including biocompatibility, bioactivity, lack of toxicity and a relatively high bioresorbability.[7] Nanocrystalline hydroxyapatite exhibits improved sinterability and enhanced densification due to greater surface area, which may improve fracture toughness as well as other mechanical properties.[22]

Elgendy et al(2015)[23] compared clinical and radiographic outcomes of nanocrystalline hydroxyapatite with or without PRF membranes in the treatment of periodontal intrabony defects and concluded n-HA is a suitable bone substitute in the periodontal treatment of intrabony defect. They proposed adjunctive use of PRF membrane in combination with n-HA bone graft resulted in clinically, radiographically, and statistically significant reduction in periodontal pocket depth (PPD) clinical attachment gain, increase bone density 6-months after surgery compared with n-HA bone graft alone.

On other hand, **Dentin chips (DC)** composed of 35% of organic materials and 65% of minerals, where it resembles bone.[25] It's being a organic–inorganic hybrid composites of proteins and minerals has got high fracture strength, hardness, and

toughness.[24] It is considered to be a suitable material for use in bone tissue engineering, since it can serve as a scaffold and a rich source of growth factors which is capable of vascularisation and angiogenesis.[64] It also contains bone morphogenetic proteins (BMPs), which can induce and increase osteogenesis.[26] It has been used in several forms including extracted noncollagenous dentin proteins, dentin particles (tooth ash) , deproteinized dentin and demineralised dentin.[90]

Lymperi et al (2015)[56] assessed the adhesion and migration of dental stem cells on human pulp ceiling cavities filled with MTA, Bio-oss, Dentin chips in an experimental model, which mimics the clinical conditions of regenerative endodontics. They found that MTA and dentin chips have a greater potential compared to Bio-Oss regarding the attraction of dental stem cells and are good candidates for bioengineered pulp regeneration.

There are various methods **to determine radiopacity of dental material and bone graft material**. Katz et al. (1990)[45], evaluated the radiopacity of gutta percha cones using an aluminium stepwedge with photodensitometer. Tanomaru-Filho et al. (2008)[94], used digitalization of the radiographic images and determining the radiopacity of materials with the aid of specific software which seems to be reliable and easily reproducible. Carvalho et al (2007)[13], on the other hand, used the sensor from the Digora system for image acquisition and the Digora software for image analysis.

In this study, we used **digital image analysis (DIGORA Software for Windows 2.9.113.490)** that has been considered of the better accuracy than transmission densitometry, providing precise and trustworthy numerical values.[81] Digora software

makes a direct capture of the radiographic image by means of a sensor that is sensitized by the x-rays, permitting a direct reading of image density with no need of previous digitization of the radiographs. The gray value of any spot indicated by the pointing device is immediately shown in pixels on the screen. The pixels already have their determined grey shades, directly providing the values at a scale 0 to 255, through the program.

Onem et al (2012)[67] compared new generation of the Digora storage phosphor plate (SPP) system [Digora Optime (Soredex, Helsinki, Finland)] with its previous version [Digora fmx (Soredex, Helsinki, Finland)]. Digora Optime achieved the best contrast at a lower exposure and demonstrated a better dose response. The perceptibility of low-contrast details was significantly higher for Digora Optime at a wide range of exposures. The spatial resolution of the Digora Optime system was reported to be 12.5 lp/mm. It was reported that the Digora Optime system provides 14-bit images, meaning that 16,384 shades of gray are available per pixel. Hence we have chosen digora optime for our study

It has been proposed that the radiopacity of restorative materials should be evaluated by using comparison with the radiopacity of the same thickness of enamel, dentin, and with an aluminum stepwedge (penetrometer) as internal standard.[81] The relative radiopacities of materials, enamel and dentin are expressed as aluminum equivalent values, in millimetres.

Stepwedges with graduated thickness and fewer steps have added benefit of speeding the measurement process.[30] So we have chosen 8 stepwedge aluminium block which was machined from a single block of aluminium alloy with each step of 1mm increase in height. ( Total length 120mm and width 10mm).

Several factors may affect the radiopacity of a material such as composition of material, amount of radiopacifier, material thickness, angulation of the X-ray beam, exposure factors (target film distance, kvp, mA, exposure time) methodology used for evaluation, type of X-ray film, and radiographic processing.[75]

Image contrast and sharpness of the material radiographed can be varied by the type and amount of radiopacifier used.[73,60] On literature search, it was found that 1-40 wt% of radiopacifiers used in root end filling material,[16,38] bone graft material[73] and in root canal sealers.[91] In our study when we tried to mix the same amount of radiopacifier mentioned above for PRF, failed to show a significant radiopacity. This might be due to the low density of the radiopacifiers used (Nano-hydroxyapatite and dentin chips) compared to metal radiopacifiers.[103]

The radiopacifiers which we used were bioactive, so tried blending 50 wt% of it with PRF. This gave satisfying results. The measured amount of radiopacifiers (50wt%) were manually triturated along with PRF, mimicked the usual procedure used in our clinical situations. Then these samples to be radiographed were compacted into Teflon rings.

Teflon rings (6mm diameter, 2mm height) were used to standardise the thickness of samples.[81] They are inexpensive, readily available in large quantities at hardware stores. Inorder to avoid errors due to non-homogenous area of sample, five density values for each samples were recorded.

The focus-film distance of 30 cm was used, as long target distances are suggested help to ensure that the X-ray sensor or film is uniformly irradiated & short exposure time (0.3 sec) was set up to reduce background fog, but short enough to visualize 1mm of aluminium alloy.[30]

The mathematical expression used in this study as suggested by Durate et al (2009)[37] represents the relation between the values of radiographic density of standard stepwedge's steps, and the thickness of aluminium. Salzedas, Louzada & Oliveira (2009)[81] demonstrated the practicity, accuracy and sensitivity of this methodology in the evaluation of bone mass variation in dogs.

However, currently no research has been addressed how biological function of resident mesenchymal stem cell, will alter when these radiopacifiers (nHA & dentin chips) used in combination with PRF. So in our study, we addressed the effect of this combination on HDPSCs proliferation, mineralization potential, protein expression for odontoblastic differentiation respectively.

DPSCs possess postnatal stem cell characteristics, including multipotent differentiation, self renewal, clonogenic capacity, expression of multiple mesenchymal stem cell markers.[35] DPSCs are also heterogenic stem cell analogous to bone marrow mesenchymal stem cells.[35]

Human DPSCs are commonly isolated from primary incisors and permanent third molars.[17] In case of third molar, the development begins at sixth year of life, the last permanent teeth that fully develop and erupt. It means that until this time, embryonic tissues from dental lamina remain quiescent and undifferentiated within the jaws, the only organogenesis event that completely occurs after birth.[17] This means that the structure of those teeth are still immature and a conspicuous pool of undifferentiated cells resident within the cell rich zone of dental pulp.[17] These cells not only proliferate significantly more than those in the mature pulp, but also have a higher differentiation capability both in vitro and in vivo.[32] It can be obtained easily as it is the common tooth extracted for routine surgical & orthodontic purposes. Based on these findings,

Human impacted third molars were selected between 20-30 years of age for obtaining pulp tissue.

**Dental pulp stem cells** can be cultured by two methods: the first is enzyme digestion method (Gronthos et al 2000)[29] in which pulp tissues are collected under sterile conditions, digested with appropriate enzymes, and then resulting cell suspensions are seeded in culture dishes containing a special medium supplemented with necessary additives incubated.

The second method for isolating dental pulp stem cells is explants outgrowth method in which the extirpated pulp tissues are cut into $2mm^3$, anchored via microcarriers onto suitable substrate, and directly incubated in culture dishes containing the essential medium with supplements.[29]Ample time (upto 2 weeks) is needed to allow a sufficient number of cells to migrate out of tissues.

Huang et al (2006)[36] compared both method and found that cells isolated by enzyme digestion had higher proliferation rate than isolated by outgrowth. Dulbecco's modified Eagle's medium (DMEM) was found to be effective to maintain cells in tissue culture. **So, in this study enzymatic digestion of pulp tissue was carried out with type I collagenase in DMEM.**

Patel et al (2009)[71] indicated that incubation of extirpated and mechanically minced rodent pulpal tissue with 0.25% trypsin:EDTA and subsequent culture in a α MEM medium provided maximal cell growth and expansion. Based on this finding passaging was carried with trypsin: EDTA solution in this study to obtain maximum cell growth.

In an in-vitro study conducted by Huang et al (2010)[34] reported that the PRF resulted in proliferation and increased protein expression of Human Dental Pulp Cells, further differentiated into odontoblasts like cells. Therefore, DPSCs are considered to be suitable cells to evaluate odontogenic differentiation in vitro.

At a minimum, scaffolds used for tooth regeneration should be biocompatible, nontoxic, non-immunogenic inorder to promote the regeneration of a single or multiple dental tissues (Yuan et al 2011)[104]. The **MTT assay** was used in our study to assess the cytotoxic effect of samples. It is considered as a sensitive and reliable indicator of the cellular metabolic activity and is preferred over the other methods measuring this end-point like the ATP and 3 H-thymidine incorporation assay, the later employing radioactivity.[72] This tetrazolium-based assay has long been regarded as the gold standard of cytotoxicity assays and has been miniaturised for use as a high-throughput screening assay.[97] Further to assess comparative evaluation of inflammation produced by samples, the level of IL-6 & IL-8 was quantified through **ELISA.**

The differentiation from dental pulp cells into odontoblasts was evaluated from the expression of genes associated with odontoblastic differentiation- **DSPP and DMP-1 and mineralization formation.**

Dentin phosphoprotein (DPP) is the most abundant noncollagenous protein and has been shown to initiate the formation of hydroxyapatite crystals within collagen fibrils. Dentin sialoprotein (DSP) is a sialic acid rich glycoprotein, which has similar amino acid composition to other sialoproteins. Dentin sialophosphoprotein (DSSP), a single transcript encoding both DPP and DSP (MacDougall M et al 1997)[57] is predominantly expressed by odontoblasts and widely accepted to be an important phenotypic marker

for odontoblast, athough it is transiently expressed in preameloblasts and bone at very low levels.

Dentin matrix protein 1 (DMP-1) is an acidic protein and has been found to be expressed in both dentin and bone. DMP-1 is a key regulator of odontoblast differentiation, formation of the tubular system and mineralization. DMP1 expression is required in both early and late odontoblasts for normal odontogenesis to proceed (Lu Y et al 2007).[55] DMP-1 which is localized in the nucleus during early differentiation of odontoblasts (Narayanan K et al 2006)[65], is able to bind specifically with the DSPP promoter and activate its transcription.

DPSCs are identified by their negative expressions of CD14, CD31, CD34, CD45, CD117, CD133 and positive expressions of CD9, CD10, CD13, CD29, CD44, CD49d, CD59, CD73, CD90, CD105, CD106, CD146, CD166.[50] Gronthos et al (2000)[29] established dental pulp stem cells by proving the expression of some perivascular markers such as STRO-1, VCAM-1, MUC-18 and smooth-muscle actin, based on their ability to regenerate, indicating that DPSCs are a heterogeneous population of mesenchymal stem cells (MSCs). The human origin of odontoblast/ pulp cells was confirmed through the expression of dentin sialophosphoprotein (DSPP) in dentin-like structures (Gronthos etal.2002).[28] It was demonstrated that STRO-1 & CD34 markers expressed more in DPSCs isolated by enzymatic digestion in comparison with outgrowth.[44] STRO-1 antibody recognizes a cell surface antigen expressed by bone marrow stromal cells and stromal precursors.[88] STRO-1 binds to approximately 10% of bone marrow mononuclear cells, greater than 95% of which are nucleated erythroid precursors.

Based on the above findings we have chosen proteins DSSP & DMP1 to assess odontoblastic differentiation and STRO-1 for characterisation of Human dental pulp stem cells. Protein expression of these were analysed using **Western blot assay**. Western blot has ability to detect as little as 0.1 nanograms of protein in a sample. Gel electrophoresis sorts a sample into proteins of different size, charge, and conformation.[58] Specific antibodies show affinity for specific proteins, the process can selectively detect a target protein even in a mixture of 3,00,000 different proteins.

**To determine the mineralisation ability of HDPCs, Alizarin red staining method** (Johnson et al 2001)[41] was chosen as it is found to be effective in determining the amount of calcium bound alizarin in various studies.

## I. ALUMINIUM EQUIVALENCY OF RADIOPACITY OF MODIFIED PRF MEASURED USING DIGORA SOFTWARE

Till now there are no research which studied radiopacity of a bio-scaffold material. This is our first attempt, where we tried making PRF a traceable material. **Salzedas Louzada, Oliveira filho etal (2006)**[81] found that Enamel showed a density equivalent to 4.19 (± 0.008) mmAl and dentin equivalent to 2.57 (± 0.0004) mmAl. In our present study, the aluminium equivalency value for Group 1– Dentin disc (2.64 ±0.020 mmAl), which was kept as a control, had maximum radiopacity compared to other 3 groups. This result was consistent with previous finding by **Salzedas, Louzada, Oliveira filho etal.** According to the American National Standards Institute and American Dental Association (ANSI/ADA) specification No. 57, endodontic filling materials should present a difference in radiopacity equivalent to at least 2 mmAl in comparison to bone or dentin.[93]

Comparing Group 3 (PRF+ 50 wt% nHA) and Group 4 (PRF+50wt% DC), Group 3 were able to achieve a radiopacity (1.51±0.089 mmAl) than Group 4 (0.97± 0.22 mmAl) , which was almost half of the Group 1 (Dentin disc-control) & the differences were statistically significant ($P<0.05$).

Laghios et al (2000)[49] compared the radiopacity of tetracalcium phosphate (TTCP) and 11 root-end filling materials relative to human dentine and found that TTCP radiopacity is 1.53 ± 0.01 mmAl, which was less than dentine .This was similar to our result.

Group 2 (PRF) and Group 4 (PRF +50wt% DC) had radiopacity value which was below the 1mm eqivalent of aluminium.

The radiopacity is mainly due to density, thicknesss of the material, energy of radiation.[95] For our study uniform thickness of each sample was exposed to x-ray & exposure factors was set up similar for each. But the density of material used varied which might have influenced the radiopacity of the sample. Generally higher the atomic number of component atoms and greater density, greater the X-ray absorbance and the larger the contrast produced.[1] The radiopacifier added was calcium phosphate based material having density (3.16 $g/cm^3$)[103] less than a commonly used metal radiopacifiers (9.79 $g/cm^3$). The nanohydroxyapatite added mainly consists mainly of inorganic component, more of calcium phosphate component whereas dentinchips are hybrid of organic –inorganic material. In PRF, the major portion is fibrin network and platelets, which are having low density.

## II. CELL CULTURE BASED ASSAYS

### 1. MTT ASSAY

An indirect cytotoxicity evaluation of the samples was conducted with the adaptation of the ISO 10993-5 standard test method.[51]The material prepared into extracts and were used in culture media. According to ISO standard 10993-5, samples are considered cytotoxic when the cell viability reduction is larger than 30%.[51]

The incorporation of bioactive reinforcements is considered as a powerful method to improve the properties of polymer. Previous studies[56,62,100] reported that these materials were not cytotoxic and hydroxyapatite improved cell adhesion.

Huang et al (2010)[34] studied the biological effects of PRF on HDPCs & found that PRF has no cytotoxic effects to HDPCs. HDPCs on the flat surface of the culture dishes demonstrated a spindle-shaped morphology. Similarly in our study also cell viability of Group B (PRF) at different concentration showed maximum cell viability/ minimum cytotoxicity when compared to Group C (PRF+50wt% nHA) and Group D (PRF+ 50wt% DC).

Comparing Group C (PRF+50wt% nHA) and Group D (PRF+ 50wt% DC), Group C had less cell inhibition rate (22%) and maintained cell viability of >75% at different concentration. Pilloni et al (2014)[74] evaluated the influence of nHA on the adhesion, proliferation and differentiation of osteoblasts. They found an increased expression of BMPs and osteoinductive biomarkers. This suggests that nano-hydroxyapatite were able stimulate the proliferation and differentiation of local alveolar osteoblasts and thus encourage bone regeneration at sites of alveolar bone regeneration.

Group D (PRF+DC) had maximum cytotoxicity, where cell inhibition was around 28% compared to other groups. Tabatabaei et al (2016)[90] studied the surface

characterization and biological properties of regular dentin (RD), demineralized dentin (DemD) and deproteinized dentin (DepD). They found deproteinized dentin showed high rate of formation/deposition of hydroxyapatite crystals and adhesion/viability/osteogenic differentiation of human dental pulp stem cells. It has been found that organic portion of dentin which has proteins can result in antigenic reaction. So deproteinizing dentin has been recommended to prevent cytotoxic reactions. Moharamzadeh et al (2008)[63] eliminated the antigenic materials in dentin by boiling the dentin powder in deionized water and refluxing in isopropanol, and obtained a mineral structure with neutral organic content. This might be reason for our study also where we used regular dentin, proteins which might have caused cytototoxic effect on HDPSCs.

2. **Enzyme-linked Immunosorbent Assay (ELISA)**

Some inflammatory cytokines involved in inflammation are tumor necrosis factor (TNF), IL-1, IL-6, IL-8, and IL-12. Although monocytes and macrophages are the main sources of these cytokines, they are also produced by activated lymphocytes, endothelial cells, and fibroblast.

**Comparing 1L-6 expression** among different groups, Group A (Control ) induced least expression of cytokine compared to Group B (PRF), Group C (PRF+nHA), Group D (PRF+DC) with $p<0.05$. There was no statistical difference between Group B (PRF) and Group C (PRF+nHA). IL-6 is a pleiotropic cytokine that has both pro-inflammatory and anti-inflammatory functions that affect processes ranging from immunity to tissue repair and metabolism.[3] Pro-inflammatory properties are elicited when IL-6 signals is transmitted via soluble IL-6 receptors binding to gp130, which is ubiquitous in all cells.[78] Inhibition of trans-signaling via gp130 blockade also leads to

recruitment of monocytes to the inflammation site, promotes the maintenance of Th17 cells (T helper cells), and inhibits T cell apoptosis and development of Tregs (T regulatory cells). In contrast, anti- inflammatory properties are elicited when IL-6 signals through the classical pathway, which occurs via the IL-6 receptor that only few cells express. IL-6 classic signaling also mediates apoptosis inhibition and the regeneration of cells.[78]

Group D (PRF+DC) showed maximum **IL-8 cytokine expression** compared to other 3 groups (Group A- control, Group B - PRF, Group C -PRF+ nHA). IL-8, also known as neutrophil chemotactic factor, has two primary functions. It induces chemotaxis in target cells, primarily neutrophils but also other granulocytes, causing them to migrate toward the site of infection. IL-8 also induces phagocytosis once they have arrived. IL-8 is also known to be a potent promoter of angiogenesis. Campos et al (2009)[12] conducted a study to analyze the location, distribution and concentration of IL-1β and IL-8 cytokines in healthy and inflamed dental pulps. Fibroblasts obtained from healthy dental pulps, stimulated in the cell cultures, to mimic inflamed pulp cells and were analyzed by ELISA for IL-1β and IL-8 they found inflamed pulps presented the higher amounts of IL-1β and IL-8 than healthy pulps. In our study, for group D (PRF+DC) we used dentin without any modifications like deproteinization. The organic component of dentin which contains acidic proteins might have caused the inflammation of HDPSCs. This lead to increased expression of IL-8 cytokine.

There was no significant difference for IL-8 expression among groups A, B and C. But existed a significant difference among Group C (PRF+nHA) and Group D (PRF+DC), where Group C (PRF+nHA) showed less expression of cytokine IL-8

compared to group D (PRF+DC). Brandell et al (1986)[10] conducted a study where they used demineralized dentin, hydroxyapatite & dentin chips as a filling material in the apical 2 mm of canals with perforated apexes in 36 anterior teeth of 8 adult cynomolgus monkeys. The amount of hard tissue formation and the degree of inflammation were evaluated after 3 and 6 months. No differences were noted between the various materials after 3 months. However, after 6 months the samples with apical plugs of hydroxyapatite had more hard tissue formation and less inflammation than the others.

A delicate balance exists between the signaling or inhibition of repair and regeneration by proinflammatory mediators. Further evidence regarding the effects of inflammation on regeneration comes from *in vitro* studies that demonstrate the biphasic responses of pulp cells to proinflammatory signaling molecules. Notably, while relatively low levels of cytokines and growth factors can be stimulatory to cells, high levels of these molecules, such as TNF-$\alpha$ and TGF-β, present during infection and inflammation can cause cell death.[3]

### 3. MINERALIZATION ASSAY

Osteoblasts can be induced to produce vast extracellular calcium deposits in vitro. This process is called mineralization. Calcium deposits are an indication of successful in-vitro bone formation and can specifically be stained bright orange-red using Alizarin red. The presence of red stained mineralized nodules on extracellular matrix was observed in all experimental groups. Highly positive stained mineralized nodule were observed in Group B (PRF), Group C (PRF+nHA) and Group D (PRF+DC).

Group C (PRF+nHA) recorded the greatest increase in the positively stained mineralized nodules.

Evidence shows that surface properties of grafts play an important role in their biological properties and function under in vitro and in vivo conditions. The increased differentiation potential of Group C (PRF+ nHA) might be attributed to the difference in their chemical composition and surface topography. nHA is similar to the natural mineral phase in dental hard tissue building unit (calcium and phosphates) and have special biological and physicochemical properties. The hydration reaction of nHA with physiological fluids results in appropriate $Ca^{2+}$ concentrations and alkaline pH (10–12) that have been shown to favor cell proliferation and differentiation forming strong mineralized interface.[62] Nanomaterials had been shown to control adhesion and differentiation of stem cells and possess superior biological properties.

Wang et al (2017)[98] developed a hybrid composite of mesenchymal stem cell (MSC) sheets with nanoscale hydroxyapatite (nano-HA) & autologous platelet-rich fibrin (PRF) granules for enhanced bone formation within a critical-sized rabbit cranial defect. The percentage of new bone in the MSC/PRF group (35.7±5.1%) was significantly higher than that in the MSC (18.3±3.2%; $P<0.05$) and empty defect groups (4.7±1.5%; $P<0.05$).

Mineralization nodule formation in Group D (PRF+DC) was less compared to Group C (PRF+nHA). This can be attributed to dentin preparation procedure. The size and shape of dentin particles play an important role in its osteoinductive and osteoconductive properties. DepD and DemD samples had a smoother and more homogenous surface than RD samples.[90] This is especially important because smoother surfaces are more suitable for proliferation and differentiation of

osteoblasts, while rougher surfaces can trigger a response by macrophages and subsequent bone loss.

**4. WESTERN BLOT**

The differentiation from dental pulp cells into odontoblasts was evaluated from the expression of genes associated with odontoblastic differentiation, such as DSPP and DMP-1. The present results showed that Group C( PRF+ nHA) & Group D (PRF+ DC) increased the protein expression of DSPP and DMP-1 of HDPSCs compared when PRF used alone as in Group B (PRF).

A previous study by Huang et al (2010)[34] showed that PRF stimulates the differentiation of HDPCs by up-regulating osteoprotegrin and ALP expression.

Mohamed et al (2018)[62] studied the effect of different bioactive materials (nHA, MTA, calcium enriched mixture) on the odontogenic differentiation potential of dental pulp stem cells using two different culture mediums and found that biomaterials promoted the odontogenic differentiation of DPSCs which might be due to their hydrophilicity together with the alkaline pH and calcium ion released as a result of their hydration reaction.

The Group C (PRF+nHA) & Group D (PRF+DC) had caused slight inflammation, but induced mineralization and odontogenic differentiation. This results was similar to study conducted by Kim et al (2017)[47] where they investigated the effects of PRF on odontoblastic differentiation in human dental pulp cells (HDPCs) treated with lipopolysaccharide (LPS). It has been shown that expression of DSPP, DMP-1, ALP activity, and mineralization were enhanced by PRF in LPS-treated HDPCs. But the role of inflammatory cytokines in the longevity and differentiation potential of dental pulp cells, it still remained a matter of controversy. However, the mechanism

underlying PRF contributing significantly to the enhanced differentiation in inflamed HDPCs remains unclear. It can be due to time-dependent slow release of anti-inflammatory cytokines from fibrin meshwork of PRF.[47]

When measured the integrated density value (IDV) to quantify the amount of proteins (DSPP & DMP-1) expressed, Group D (PRF+ DC) showed highest value compared to other groups. Liu et al (2015)[52] conducted a study to investigate the effect of demineralised dentin matrix (DDM) on dental pulp stem cells (DPSCs) and the potential of complexes with DPSCs and DDM for mineralized tissue formation. Mineralized tissue formation was observed with the DPSC & DDM and DPSC & HA-TCP (hydroxy-apatite – tricalcium phosphate) combination. The mineralized tissue of the DPSC + DDM combination stained more positive for DSPP, similar to the dentin tissue.

STRO-1 expression was used in our study for characterization of human dental pulp stem cells. It has been shown that STRO-1 expression was higher in Group C (PRF+nHA), Group B (PRF), Group D (PRF+DC) when compared to Group A (Control). This suggests that addition of PRF and modified PRF were able to induce proliferation of immature HDPSCs. Stewart K et al (1997)[88] demonstrated the expression of STRO-1 in cultures of BMSCs and in established human osteosarcoma cell lines in vitro and concluded that its expression was characteristic of less well differentiated cells.

Within the limitation of the present study, we have shown that addition of bioactive radiopacifiers – Nano-Hydroxyapatite (nHA) and Dentin chips (DC) were able to impart radiopacity to the PRF. But radiodensity provided by Nano-Hydroxyapatite group was better than dentin chips. Both the bioactive radiopacifiers are non-

cytotoxic, induced mineralization and odontogenic expression of HDPSCs. These results of our study proved that these bioactive materials along with PRF are having promising properties to serve the best for the future.

For better properties, these radiopacifiers can be modified further. It has been shown that different reaction temperatures can influence the size and crystallinity of nHA on osteoblast viability.[54] Dentin chips preparation can also be modified as it plays an integral role in its osteoinductive and osteoconductive properties. And further experimental studies are needed to elucidate the anti-inflammatory effect of PRF on the regulation of odontoblastic differentiation.

**SUMMARY**

The ultimate goal of a regenerative treatment strategy in endodontics is to induce regeneration of pulp - dentin complex. To accomplish this, one needs a scaffold which can provide a 3D environment for cells to attach and grow. It should be able to orchestrate and differentiate the homing of endogenous cells. Inorder to achieve this, precise placement of a scaffold material is needed especially in case of necrotic immature tooth. The chemical and physical properties provided by the scaffold should be same as natural surrounding tissue as far as compatibility, adhesion, migration, proliferation, degradation is concerned.

The platelet concentrate, PRF which we used in study was able to statisfy criteria for an ideal scaffold except its lack of radiopacity. Our study made PRF a traceable scaffold by adding 50 wt/% of nHA & 50 wt% of DC. We compared this modified PRF'S aluminium equivalent of radiopacity & it's effect on HDPSCs, the a) cytotoxicity b) IL-6 & IL-8 cytokine expression c) mineralization potential d) DSPP, DMP-1, STRO-1 expression.

The groups were divided into 4 as follows

**For measuring aluminium equivalent of radiopacity**

Group 1- Dentin disc (control)

Group 2- PRF

Group 3- PRF+ 50 wt% nHA

Group 4 – PRF + 50 wt% DC

**For cell culture based assays**

Group A- DMSO (Control)

Group B- PRF

Group C- PRF+ nHA

Group D- PRF+DC

DIGORA software for windows 2.9.113.490 was used to measure the radiodensity of the samples and was converted to aluminium equivalent of radiopacity. The Group 3 (PRF+ 50wt% nHA) was able to achieve significant amout of aluminium equivalent of radiopacity 1.51± 0.089 mmAl than Group 4 - PRF+50wt% (0.97±0.22 mmAl). Also it is observed that aluminium equivalent of radiopacity for Group 3 (PRF+ 50wt% nHA) was almost half the density of Group 1- Dentin disc (2.66±0.02 mmAl).

The MTT assay for cytotoxicity showed that samples ,Group B (PRF), Group C (PRF+ nHA), Group D (PRF+DC) maintained the cell viability > 73% .

To assess the inflammation induced by the samples, IL-6 and IL-8 cytokine expressions were quantified by ELISA. IL-6 & IL-8 expressions was higher in Group D (PRF+ DC) compared to Group C (PRF+nHA) & Group B ( PRF).

Alizarin red staining was performed to detect amount of mineralization induced. Group C (PRF+nHA) showed appreciable amount of mineralization nodules when viewed under phase contrast microscope. In Group B (PRF) and Group D (PRF+DC) the no: of mineralization nodules per field viewed was less.

DSPP, DMP1 AND STRO-1 protein expression were analysed through Western blot assay. Density of protein bands formed were measured by a Image J software. It is expressed as Integrated Density Values (IDV). The odontogenic expression evidenced by DSPP and DMP1 proteins, were higher in Group D (PRF+DC) followed by Group C (PRF+ nHA) when compared to Group A (PRF).

The order of the amount of STRO-1 expression given by the groups as follows: Group C (PRF+ nHA) > Group B (PRF) > Group D (PRF+DC). This suggests that addition of PRF and modified PRF were able to induce proliferation of STRO-1 positive immature HDPSCs.

## CONCLUSION

Within the limitations of the present study it can be concluded that:

- The calcium phosphate based bioactive material nano-hydroxyapatite were able to impart radiopacity better than dentin chips making PRF as a traceable material.
- Both the bioactive materials used in the study are non cytotoxic
- The combination of these bioactive material with PRF has got synergestic effect. It enhanced the mineralization and odontogenic differentiation of HDPSCs than when PRF used alone.
- The addition of bioactive materials (nHA & DC) in PRF had caused slight inflammation of HDPSCs. But were able to induce mineralization and odontogenic expression after 14 days, suggestive that PRF has got anti-inflammatory properties. The osteoblastic –odontoblastic differentiation of HDPSCs were evidenced by presence of mineralization nodules, DSPP and DMP-1 protein expression. Further studies at molecular level are needed to prove this.
- The increase in STRO-1expression by the groups indicates that addition of PRF and modified PRF can induce proliferation of immature HDPSCs.

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